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THIOPHOSPHATE POISONING IN WHITE PEKIN DUCKS

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Received 1 Nov 1956

THE control of flies is a major problem on commercial duck farms. A majority of the farms have government-inspected dressing plants as an integral part of the operation, and these must be maintained relatively free of flies. To this end a constant effort is made to control flies in the buildings where the ducks are raised. DDT and Lindane have had decreasing effectiveness against flies; therefore, one of the larger farms used a thiophosphate spray (Diazinon) in August 1954.

The farm in point raises about 200,000 ducks annually and housed approximately 30,000 ducks of all ages at the time of spraying. In all, pens housing 18,579 ducks were sprayed. The Diazinon was mixed for poultry as directed by the manufacturer (32 pounds of Diazinon 25 W per 100 gallons of spray). The walls and ceilings of the buildings were wet down with a power sprayer. The youngest ducklings (8 and 15 days old) were confined, the older groups (22, 29, and 36 days of age) were driven into open yards during the spraying and then allowed to re-enter the houses.

Starting with the pen holding the youngest ducks, the spraying was accomplished in about 2 hours. Mortality started in the youngest group by the time spraying was completed, and an hour later 100 percent of 3,200 eight-day-old duck-

lings and 100 percent of 3,653 fifteen-day-old ducklings died. The mortality in three older lots (11,726 ducklings) was 50 percent in 5 hours, and an additional 25 percent died within the next 24 hours.

Forty-three birds of various ages were collected for post-mortem examination. The young ducklings died a few minutes after symptoms were noticed. The affected ducklings in the older age group survived for a longer time. The only symptoms were inability to stand and tremors of the head and neck. Macro- and microscopic lesions were limited to congestion of the lungs. Fly larvae were frequently found in the gizzards of the older ducklings.

Liver, lung, kidney, and gastrointestinal contents were collected for toxicological examination. Alkaline hydrolysis followed by diazotization with sulfanilic acid failed to reveal the presence of a thiophosphate derivative. However, similar tests run on known normal and poisoned birds (laboratory ducks poisoned with Diazinon) were also negative for thiophosphate.

Blood was drawn from moribund birds for cholinesterase determination. Cholinesterase was lowered to 67 percent of normal in one duck, but other moribund ducks had normal cholinesterase levels.

A flock of 50 chickens on the same farm was sprayed with the same Diazinon mixture with no untoward effects.

The urgent need for an effective fly spray for duck farms made it essential that an insecticide less toxic than Diazinon be found. Studies of a supposedly less toxic, but effective thiophosphate (Malathion) were undertaken.

EXPERIMENTAL DATA

Six-week-old White Pekin ducklings, each weighing approximately 2 kg, were used in all tests. The insecticides were administered per orum and per trachea as presented in table I.

The per orum lethal dose is approximately 1,100 mg/kg (in ducks) for Malathion as compared to 14 mg per kg for Diazinon. Intratracheally the lethal dose for Malathion was 600 mg per kg and for Diazinon about 6mg/kg. Malathion was

further tested under field conditions as follows: Ten ducklings 8 weeks old were individually dusted twice (3 days between dustings) using 1 pound of a 25 percent Malathion dust for each application. A second lot was dusted similarly with 4 percent Malathion. The ducks were hand-dusted with a small mechanical duster. As much of the dust as possible was worked deep into the feathers. No mortality or loss of weight occurred. Similar lots of ducklings were hand-sprayed twice (3 days apart) with a 5 percent and an 0.5 percent aqueous solution of Malathion. Each duckling received approximately 60 ml of the solution applied directly to ruffled feathers.

TABLE I

The effect of Malathion and Diazinon administered per orum and per trachea to 6-week-old White Pekin ducklings

Mg of insecticide	Malathion		Diazinon	
	Per orum	Per trachea	Per orum	Per trachea
4,560.0	5/5*	5/5		
2,280.0	4/5	5/5		
1,140.0	0/5	2/5		
570.0	0/5	1/5	5/5	5/5
285.0	0/5	0/5	5/5	5/5
28.5	0/5	0/5	3/5	5/5
2.8	0/5	0/5	0/5	2/5

* Numerator = mortality. Denominator = number of ducklings dosed.

Three lots of 25-day-old ducklings were started on three different litters (straw, shavings, and sugar cane pulp) which had been treated with 20 pounds of 4 percent Malathion dust per 100 square feet of floor space. The litter was replaced with new treated litter at weekly intervals for 4 weeks. Weight gains were normal and there was no mortality.

SUMMARY

1. A case of thiophosphate (Diazinon) poisoning causing an 84 percent mortality in 18,579 ducklings 1 to 5 weeks of age is described.

2. Diagnosis by lesions or clinical laboratory tests is difficult if not impossible.

3. The per orum lethal dose for Malathion is 1,100 mg per kg and for Diazinon is 14 mg per kg in White Pekin ducklings. The intratracheal lethal dose is 600 mg per kg for Malathion and 6 mg per kg for Diazinon.

4. Ducklings dusted, sprayed, or raised on litter treated with several times the recommended levels of Malathion were not adversely affected.

5. Insecticides considered safe for chickens or other gallinaceous birds should be revaluated for ducks.

AN ARTIFACT RESEMBLING FOCAL NECROSIS IN THE LIVERS OF SLAUGHTERED WHITE PEKIN DUCKS

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Received 10 May 1957

FOR THE past two years inspectors in the eviscerating plants on Long Island have been condemning a significant number of duck livers because of the presence of diffuse,

"necrotic" areas in the right and to a lesser extent, the left lobe of the liver. In view of the increasing number of condemnations, it was decided to investigate the nature and cause of the liver abnormality.

EXPERIMENTAL

Studies were made on a commercial flock of 2,500 eight-week-old ducklings processed through a commercial dressing

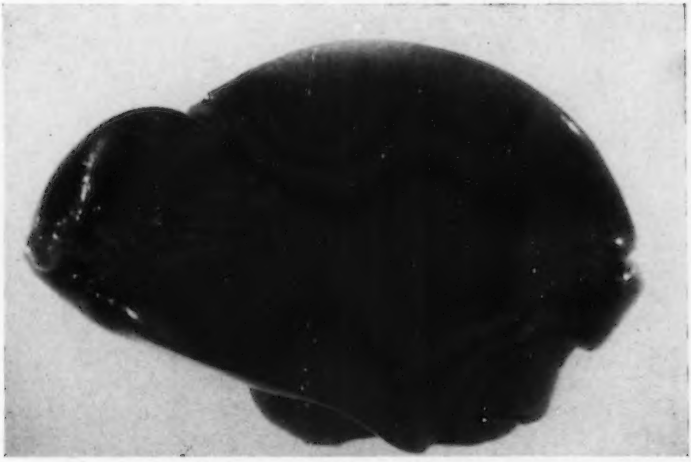

Fig. 1. Medial (cardiac) aspect of right lobe of liver with whitish spots (artifacts resembling focal necrosis) in commercially slaughtered and picked ducks (X2).

plant. The ducks in question were vigorous, healthy birds of the White Pekin breed, slaughtered at 7 to 8 weeks and weighing approximately 6 pounds. All liver specimens were collected on the eviscerating line at a local processing plant. A similar condition was observed in all eviscerating plants in the area. The livers of such ducks normally are rather soft, tender and contain a variable amount of fat, (from 0 to 80 percent on a dry weight basis). On gross examination the affected livers contained focal necrotic appearing areas about 1 mm in size often coalescing to form grape like clusters (figure 1). The

areas were white to yellowish-white in color and were located most often in the right lobe of the liver in that portion which is in contact with the heart. When incised, they often appeared to be around a blood vessel. Ducks with soft, fatty livers appeared to have more severe damage than those with more normal livers. This type of liver damage has not been seen in ducks submitted to the laboratory by duck farmers for postmortem examination or in ducks that had been picked by hand.

Microscopically, the areas of affected liver had a focal distribution and varied in size from a few cells up to several lobules. They often appeared to involve areas adjacent to vessels, either central (efferent) veins or portal vessels. The centers of many of the areas were acidophilic with all cellular detail missing. Within this acidophilic material there were small, granular, amorphous, basophilic masses which took a Wright's stain and appeared to be nuclear dust rather calcification. Outside the acidophilic area there was a narrow zone of liver cells with normal staining cytoplasm but varying degrees of nuclear disintegration and dissociation of the parenchymal cells. Toward the periphery there were cells with nuclei in various stages of karyorrhexis and karyolysis. The margin of the area was indistinct and vaguely defined (figure 2). There was a remarkable absence of cellular and vascular reaction as might be expected around necrosis. The general impression was one of focal areas of damaged, degenerate-appearing liver cells, without evidence of reaction. The lack of reaction was surprising in view of the fact that liver necrosis of infectious origin (duck virus hepatitis) is accompanied by both heterophils and mononuclear cell types with active regeneration beginning in a day or two. The absence of leukocytic or regenerative reaction in the affected livers indicated that this liver change occurred near the time of death of the bird. In addition, the focal distribution, vague boundaries, and general cytology suggested that the change might have been agonal, possibly intensified by postmortem autolysis.

Attempts to demonstrate an infectious agent by intraperitoneal inoculation of ground affected liver into seven week old

ducklings were unsuccessful. Cultures on infusion agar, PPLO agar, blood agar, duck infusion broth, tryptone broth and inoculations of embryonating duck and chicken eggs yielded nothing.

Observations indicated that the liver artifacts did not exist or develop in bled out ducks that had not been scalded or picked. Furthermore, liver changes were not observed in scalded ducks that had not been put through the automatic, rotary,

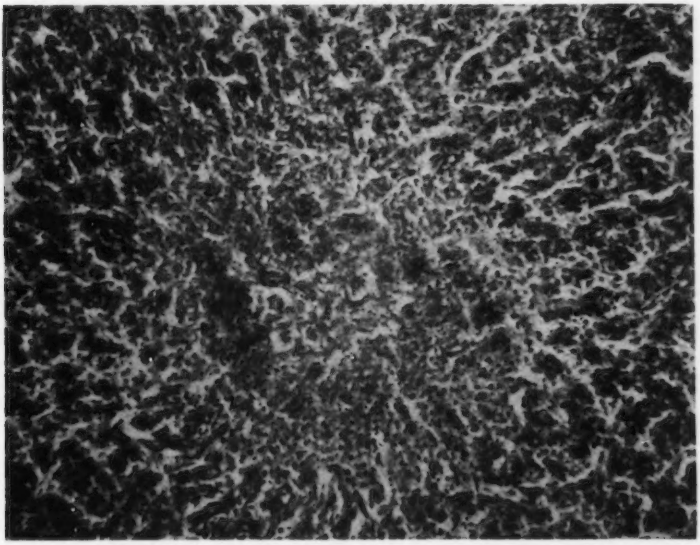

Fig. 2. Histologic section of the liver artifact. Note extensive loss of architecture and cellular detail. The nuclei in the involved area are disrupted or absent. There is considerable amorphous, acidophilic debris in the area. Note the complete absence of cellular reaction. Hematoxylin and eosin (X72).

picking machine. This liver damage developed during the two hours following feather removal for 50 seconds in the rotary batch picker. The rotary picking machine consists essentially of a rapidly whirling horizontal disc with rubber, finger-like projections inserted vertically. This forms the bottom of a cylinder from the stationary walls of which extend additional rubber, finger-like projections and a series of low, longitudinal,

steel baffles. Seven to 8 ducks are placed in the machine at one time. The rotary disc spins the ducks violently against one another and against the fingers on the side of the cylinder causing the feathers to be removed by friction. This is described as a free tumbling action. Because severe damage to the skin and flesh can be caused in this machine, 50 seconds is the maximum time permitted for defeathering. The extent of liver damage was no more severe even after 10 minutes in

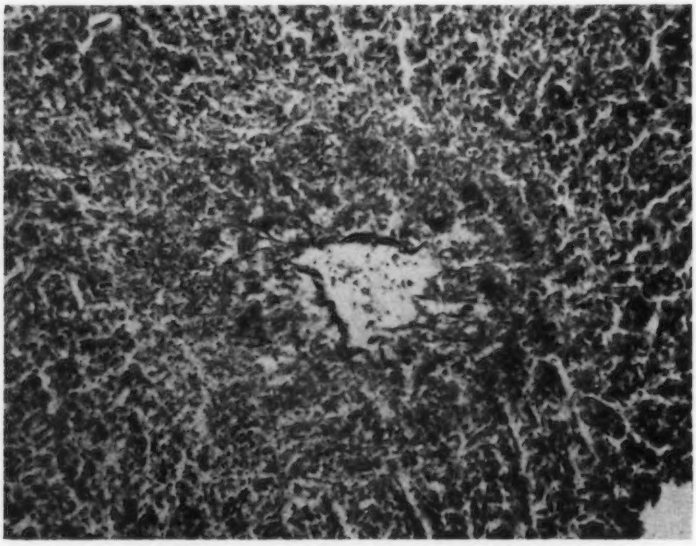

Fig. 3. Histologic section of liver artifact produced experimentally by injecting bile into the parenchyma of a normal isolated liver. The histologic appearance is similar to that in figure 2. Hematoxylin and eosin (X72).

the machine. The liver damage reached its maximum severity within 2 hours after feather removal.

Duck bile collected from the gall bladder of eight-week-old ducklings was injected into the parenchyma of normal livers from freshly bled-out ducks that had not been through the rotary picker. The bile was deposited just under Glisson's capsule along a two inch tract made by a 23 gauge needle. After 2 hours at room temperature, portions of the liver along

the site of injection were examined grossly, fixed in formalin and later sectioned. The gross change was similar to the liver damage caused by the batch picker, except that where small pools of bile had been injected, the liver had a greener color. Microscopically, the bile produced changes indistinguishable from the liver artifact caused by the picker (figure 3).

DISCUSSION

The liver artifact noted at evisceration, probably was caused by injury to the small bile ducts at the time of picking. This injury could have resulted easily from the repeated impact of the soft liver tissue against the adjacent hard surface of the heart, while the duck carcass was being violently thrown about in the picker. Repeated failures to demonstrate an agent infectious for ducks from affected livers gave further support to the concept that the liver changes were artifacts. This type of liver damage in the absence of infection does not constitue a human health hazard.

ACKNOWLEDGMENT

The authors gratefully acknowledge the assistance of Dr. Daniel DeCamp and Dr. Roger E. Dodge of the Poultry Inspection Section, United States Department of Agriculture in securing material for this study.

SUMMARY

1. A non-infectious abnormality of the liver in the White Pekin duck in the form of whitish spots on the medial, cardiac portion of the right lobe is described.

2. This liver damage was not present in ducks which had been hand picked. Ducks dressed in rotary, batch pickers developed the abnormality during the two hours following picking.

3. Injections of normal bile into normal livers of freshly killed ducklings produced similar damage.

4. It is concluded that the liver abnormality is an artifact probably induced during the machine process of feather picking wherein the duck carcass is handled quite roughly. Probably rupture of the small bile ducts releasing bile into the parenchyma is responsible for this condition.

EFFECTS OF INFECTIOUS BRONCHITIS ON THE REPRODUCTIVE TRACTS, EGG PRODUCTION, AND EGG QUALITY OF LAYING CHICKENS*‡

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INFECTIOUS bronchitis is a virus disease affecting the respiratory system of both young and mature chickens. The disease was first observed in chicks in North Dakota by Schalk and Hawn.[14] Since that time, the disease has become widespread. In the northeastern United States, infectious bronchitis now exists primarily as a problem in adult laying flocks. Though the effects of infectious bronchitis in chickens on egg quality and production have received much attention, associated changes in the reproductive tract have not been extensively investigated.

The disease is not transmitted through the egg. All infections naturally acquired are postembryonic. Natural exposures occur by inhalation or ingestion of the virus. The incubation period of infectious bronchitis in fully susceptible birds is about 18 to 36 hours. The disease spreads rapidly among birds in a flock and is manifest by coughing, sneezing, and moist rales. Symptoms persist usually for two weeks.

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* Except for the data on histopathology of the ovaries, this is a condensation of a thesis which was presented by the senior author to the faculty of the Graduate School of Cornell University in September 1954 as a partial fulfillment of the requirements leading to the degree of Master of Science. Histopathological studies of the ovaries, contribution #1062, were conducted at the Massachusetts Agricultural Experiment Station, University of Massachusetts.

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‡ This project was supported in part by funds from ARS NE5 project.

Infections in chicks may occur very soon after hatching with a resulting respiratory disease and often a considerable mortality. Broadfoot *et al.*[2] reported a natural outbreak of infectious bronchitis in some 14,000 susceptible pullet chicks in 35 flocks during the first 5 to 7 days of life. The chicks, a high producing strain, suffered death losses varying from 5 to 35 percent. Later at the age of 7 months, none of these flocks were producing at more than a 50 percent rate. Many thousands of birds of identical parentage, but having suffered no respiratory disease, were laying at the normal rate (70 to 90 percent). The implications of this report are that infectious bronchitis in very young chicks may permanently damage the reproductive system of certain birds or may injure the reproductive tract of all birds to a lesser extent.

In mature birds, the most serious economic loss from infectious bronchitis is reflected in egg production. Van Roekel *et al.*[18] reported that a rapid decline in production may occur together with marked alterations in exterior and interior quality of the egg, which include misshapen appearance, rough and thin shell, bleached shell color, and watery albumen. Recovery of egg production to preinfection levels rarely occurred. Too often, abnormal shell quality and shape continued for indefinite periods. A typical production pattern is that output falls to near zero within 2 weeks after the appearance of the disease. The level of production continues to remain rather low for about another month. About 9 weeks after the appearance of the disease, production reaches a 50 percent level and is maintained there.

In a report by Urban and Goodwin,[16] pullets that were affected when they were about ready to lay, suffered the greatest decline in egg production and deterioration of egg quality. They reported that outbreaks in pullet chicks older than brooding age suffered a greater reduction in egg size than the controls.

Postmortem findings reported by Urban and Goodwin[16] indicate that impacted oviducts in some birds that had been affected with infectious bronchitis was not an uncommon finding. Broadfoot *et al.*[2] reported the finding of underdeveloped oviducts and cysts attached to the oviducts of some of the

birds that had suffered from infectious bronchitis at an early age.

MORPHOLOGICAL DIVISIONS OF THE FEMALE REPRODUCTIVE SYSTEM

The reproductive tract of chickens consists of the ovary and the oviduct. The ovary is a mass of loosely connected vascular, yellow, and rounded follicles. All follicles grow from the cortex, which surrounds the vascular medulla except at the hilus. Each developing follicle is made up of an ovum enveloped by the membrane granulosa and the thecae interna and externa. The oviduct of the hen is divided into five more or less delimited regions. Beginning at the anterior end of the tract, Surface[15] has designated the various parts as (1) the funnel, (2) the albumen secreting region, (3) the isthmus, (4) the uterus or shell gland, and (5) the vagina. In cross sections of the oviduct there are seven layers of tissue. From the outside in, these are (1) the serous epithelium, (2) a layer of outer longitudinal muscular fibers which are continuous with the musculature of the ventral and dorsal ligaments of the oviduct, (3) a layer of connective tissue containing large blood vessels, (4) a layer of inner circular muscle fibers, (5) another connective tissue layer containing some smaller blood vessels, which in this paper will be referred to as the lamina propria, (6) a layer of highly glandular tissue (tubular glands), and (7) the inner lining of epithelial elements.

MATERIALS AND METHODS

White Leghorn chickens, 7 months old, were obtained from a bronchitis susceptible closed flock maintained at the Poultry Disease Research Laboratory Farm of the New York State Veterinary College. One hundred twenty-six birds in full production were carefully selected from a group of about 225 pullets. These birds were weighed, banded, and arranged in three groups. Each bird in one group had a matching bird in each of the other groups with no more than 0.5 pound weight difference.

To establish the absence of infectious bronchitis antibodies and Newcastle hemagglutination-inhibition (HI) an-

tibodies in the birds prior to experimental use, blood samples were taken from 20 birds as representative of the entire group. The serum-neutralization (SN) test for infectious bronchitis[5] and the HI test for Newcastle disease[4] were negative in all the birds tested. Tracheal mucus from four birds of this same group was cultured in egg embryos to test for the presence of the chronic respiratory disease agent.[19] These were all negative.

The birds were placed in laying batteries in adjacent virus isolation units.[6] Forty-two birds were used in each of the three groups. Group I was inoculated with infectious bronchitis virus. At the height of the respiratory infection, birds whose rate of production and egg quality were most severely affected were sacrificed. Gross and microscopic examinations of the oviduct tissues were made. When recovery from the disease was taking place, additional birds were sacrificed for study. Infectious bronchitis is always accompanied by a decrease in feed and water consumption. It is well known that a drop in feed and water intake by themselves will have a marked downward effect on egg production. For this reason, birds in a second group, group S, were not infected with the virus but were subjected to physiological stress by withdrawing all water and feed for 3 and 4 days, respectively. The reproductive tissue of these birds were also collected for study. The third group of birds, group C, served as normal controls. They were neither subjected to infection or stress.

At the end of the experimental period of 5 months, SN tests for infectious bronchitis and HI tests for Newcastle disease were repeated on four blood samples from each group. All blood samples in group I were positive to the SN test and negative to the HI test. All blood samples in groups S and C were negative to both tests. Egg embryo inoculations with material from swabs of two tracheas from each group did not yield the chronic respiratory disease agent.

The virus used for the infection of group I birds was a mixture of Bickhart, Yacowitz, and VFR. strains. Only bacteria-free virus that was further treated with penicillin and streptomycin was used for the infection. The virus was di-

luted 1:5 and was sprayed over the face of each bird with a nebulizer. About one-half of the birds came down with respiratory symptoms of sneezing, gasping, and rales at the end of 24 hours. The rest showed symptoms at the end of 48 hours. Unfortunately, 10 days after the experimental infection of group I, respiratory symptoms appeared in group S. Likewise, group C had respiratory symptoms after 15 days. Tracheal mucus from birds of groups S and C yielded infectious bronchitis virus by chick embryo cultivation.

Five and six birds had been taken from groups S and C, respectively, for tissue studies of the oviduct at least 36 hours before the occurrence of respiratory symptoms. The remaining birds in these two groups were discarded. Fortunately, sibs of the same age were available. Eighteen and 16 birds were selected to constitute the replacement groups S and C, respectively. These birds were placed in the same type batteries used originally but were housed at the Poultry Disease Research Laboratory Farm. This situation created an 8-week-time difference between the collection of data from group I and the new groups S and C.

Egg production records were kept for each bird. After the birds were placed in individual cage batteries, periods of 7 and 5 weeks were allotted for group I and groups S and C, respectively, to become accustomed to their environment before beginning the experiment. A complete commercial ration of pelleted feed was fed to all three groups of birds. Oyster shells and grit were fed free choice at all times. Eggs were collected daily and placed in a cool room (59 F) until the following morning when they were weighed in ounces per dozen. Also at this time, internal egg quality tests were done. Egg quality was established for each bird in all groups just prior to subjection to infection or stress. In the infected birds (group I) egg quality determinations were made daily, postinoculation. In groups S and C egg quality tests were reported 7 weeks after stress.

Egg quality was measured in the following way: (1) Egg-shape index was established by dividing the width of the egg by its length.[8] (2) The internal quality was measured

in terms of the average height of the firm albumen and expressed in terms of Haugh units.[9] (3) The internal quality was also determined[17] by using the criteria as pictured in the chart on "Internal Quality of Egg." (4) A record was kept of meat and blood spots and other material in broken-out eggs. (5) The thickness of the egg shell half way between the ends of the egg was measured in thousandths of an inch with an Ames thickness measure.[7] These measurements could not be made on soft-shelled eggs.

Tissues for microscopic study were taken from birds selected on the basis of sets. A set was made up of one bird from each of the three groups. Birds selected from groups I and S were matched when their individual egg production rates were similar. This applied to birds out of production as well as in production. To complete a set, a control bird in full production was selected from group C. It is granted that other factors such as size or weight of birds may have had some influence on the size and weight of the reproductive tract. However, it was felt that the physiological status of the birds had the greatest influence on the gross and microscopic structure of the reproductive tract.

The oviducts were trimmed of their ligaments, weighed, measured, and then slit along the dorso-longitudinal aspect. This enabled a rapid and effective exposure of the oviduct surfaces as they were fixed in 10 percent formalin with 1 percent calcium acetate buffer. The container used for holding the fixative was a stainless steel covered tray (30 by 16 by 4 inches) with a layer of wax over the bottom. This enabled the pinning of tissues in a flat position to prevent curling and contraction at the time of fixation. Fixation of the oviduct was allowed to take place for 48 hours in the tray after which the whole oviduct was stored in quart jars containing the same type of fixative. The ovaries were also fixed and stored in quart jars.

Twenty-four reproductive tracts from group I, ten from group S, and seven from group C were sectioned. Cross sectional portions of ovarian tissue and associated follicles were taken. Transverse segments of the oviduct were taken at the

levels of the anterior and posterior regions of the funnel; the anterior, middle, and posterior regions of the isthmus; the anterior and posterior regions of the uterus; and the middle portion of the vagina. Portions of the tissues for sectioning were cleared in dioxane for 18 hours and then infiltrated with paraffin for 4 hours. The sections were cut 6 microns thick. Two to four sections from each level of the oviduct were stained with hematoxylin and eosin.[11]

RESULTS

Egg production and egg quality. Most of the birds in group I went out of production about the 5th day after inoculation, and only two birds of the group of 36—six had already been sacrificed—were still laying 10 days after being infected. During this period, water and feed consumption had decreased to approximately one-third and one-half respectively of the normal intake. A gradual return to normal feed intake took place during the next 2 to 3 weeks. From 3 to 5 weeks after the onset of the infection, about 30 percent of the birds of group I came back into production at an average rate of better than 50 percent, most of them at a considerably higher level. The remaining 70 percent of the birds of group I had not returned to production at the end of the 5th week of infection. The average period of time that all of the birds in group I were out of production was 35 days.

One bird went out of production 4 days after inoculation and did not lay any eggs to the time of sacrifice 51 days later. Its comb and wattles were well developed, bright red and waxy in appearance. Its whole beak, eye-ring, and shanks were bleached. The vent was oval, moist, bleached, and enlarged. The abdomen was full, soft, and pliable. The pubic bones were well spread apart. Throughout the entire period of time, this bird had every appearance of a laying bird. This type of bird has been termed a "false layer."

Birds in group S dropped from a 67 percent production rate to zero in 4 days when placed under physiological stress of thirst and starvation. Normal consumption of feed and water was established as soon as their rations were restored. From the 11th to the 19th day after stress, every bird came

back into lay at better than a 54 percent rate. The average period of time that all of the birds of group S were out of production was 11 days.

Since nothing was done to upset the birds of group C, normal production was maintained.

In table I, group average egg weights taken just prior to inoculation or stress are compared to those taken within the same group 7 weeks later. Only data available at both times from individual birds were used.

TABLE I

Average gain or loss in ounces per dozen of eggs 7 weeks after inoculation or stress

Group*	No. of birds	Gain or loss in ounces
I	23	—0.46
S	14	+1.76
C	9	+1.06

* Group I data 7 weeks after inoculation. Group S data 7 weeks after stress. Group C data at same time as group S.

A comparison of eggs in each grade just prior to inoculation or stress and 7 weeks later is given in table II.

TABLE II

Distribution of eggs by weight grades before and 7 weeks after inoculation or stress

Group*	No. of birds	Percentage of eggs by weight grade							
		Pre-inoculation or —stress				Post-inoculation or —stress			
		EL†	L	M	S	EL	L	M	S
I	23	4.0	57.0	35.0	4.0	9.0	39.0	43.0	9.0
S	14	7.0	57.0	22.0	14.0	43.0	36.0	21.0	0.0
C	9	11.0	45.0	44.0	0.0	22.0	45.0	33.0	0.0

* Group I data before inoculation and 7 weeks later. Group S data before stress and 7 weeks later. Group C data at same time as Group S.
† EL = extra large, 27 plus oz. per doz; L = large, 24 to 27 oz; M = medium, 21 to 24 oz; S = small, 18 to 21 oz.

In analyzing the data in tables I and II, one finds an average weight gain of 1.76 and 1.06 ounces per dozen of eggs

in groups S and C, respectively, as a result of certain individual birds laying larger eggs. This is a common phenomenon in normal laying flocks as they go through a laying year. In the infected birds, instead of a gain in the weight of eggs, there was a slight loss of 0.46 ounce per dozen. Some birds were mildly affected inasmuch as they either continued to gain or were able to hold the egg weight. In other more severely affected birds, the effect of the infection on egg size was so severe that it caused a lack of gain or an actual loss of egg weight.

Internal egg quality. Egg quality data were established for each individual bird in all groups prior to inoculation or stress. After inoculation, eggs were broken out daily onto a glass plate where the eggs were observed and measured for egg quality as previously described.

Table III below presents a comparison of the three groups of birds in relation to internal egg quality. Though one-fourth of the birds in groups S and C produced eggs of one score unit lower in quality, the rest of the birds in these two groups were able to maintain their quality. This is not unusual in a normal laying flock. Within group I, however, nearly two-fifths of the birds had lower scores of three or more units in internal egg quality.

TABLE III

Changes in internal egg quality 7 weeks after inoculation or stress

		Percentages of birds with						
		Increase in quality units		Decrease in quality units†				
Group*	No. of birds	One	No change	One	Two	Three	Four	Five
I	23	9.0	22	30	....	13	22	4
S	14	....	79	21	....	....	....	..
C	9	....	78	22	....	....	....	..

* Group I data 7 weeks after inoculation. Group S data 7 weeks after stress. Group C data at same time as Group S.
† Unit values based on U.S.D.A. score unit.

There was no significant increase in the size or frequency of either meat or blood spots in eggs from the infected over

the noninfected groups. In this study, meat and blood spots were noted but not used in determining egg quality.

Certain infected birds produced eggs with clumps of soft, white, granular masses up to 1 cm in diameter in the firm albumen. This condition occurred in 20 percent of the infected group beginning about 6 weeks after inoculation. However, the occurrence of this abnormality was intermittent with the frequency ranging from 5 to 50 percent of the eggs produced by the involved birds. Chickens producing eggs with granular masses also laid eggs with a significant decrease in the height of the albumen as compared with eggs laid during the preinfected period.

Egg-shape index. Although the techniques used in establishing egg-shape indexes did not reveal significant changes resulting from the infection of group I, seven group I birds laid eggs of which 5 to 50 percent were misshapen. The misshapen appearance resulted from only portions of the shell lacking their normal curvature. These portions of shells were often thinner than the adjacent shell and were located most frequently between the plane of the greatest width and the pointed end of the egg. Occasionally a misshapen egg was flat-sided. The same abnormal shape was usually produced by individual birds. Groups S and C had no significant difference in egg-shape index, neither did they produce misshapen eggs.

Shell thickness. Shell thickness was another property which appeared to be severely affected primarily in certain infected birds. Twenty-five percent of the birds of group I laid soft-shelled eggs consistently, and another 6 percent laid them intermittently. In the latter group, the egg shells when present were quite thin. Egg shells less than 32/1,000 of an inch were considered thin. Of the infected flock, 12.5 percent were laying thin-shelled eggs at 7 weeks after inoculation.

Egg shells from 10 percent of the infected birds had white, verrucous, calcareous deposits on the outside surface. These deposits ranged up to 0.5 cm in size, and their location on the shell was the same for each bird each time they appeared.

Shell thicknesses of eggs from groups S and C showed no significant change.

Gross pathology of the reproductive tract. Reproductive tracts of the control birds had no gross changes during the period of time when the infected birds of group I were being sacrificed. Measurements of oviducts and ovaries fell into the following ranges:

Oviduct weight	42.5 to 58.5 grams
Oviduct length	21.5 to 30.0 inches
Ovary weight	41.5 to 72.5 grams

Regressive changes were seen in the reproductive tracts of birds of group S from 5 to 15 days after the beginning of the physiological stress period. On the 8th day the oviduct of one bird decreased in size to as little as 19 grams in weight and 15 inches in length. Upon return of the birds to their normal feed consumption, recovery of the oviducts to normal weight and length took place in 11 days. Seven days after the beginning of stress, ovaries had regressed to as little as 8.3 grams. Ova that had reached the greatest development had the greatest degree of regressive changes. Many of the ova were shrunken, wrinkled, and "fig-shaped;" some were dark-red or dark-brown; some had a lack of continuity of the yolk membranes, which allowed the leakage of yolk material into the peritoneal cavities; a few appeared normal in shape and color. Observations made of the peritoneal cavity showed the presence of a yellow-orange, watery fluid within 5 days after the beginning of stress. The quantity of fluid from any one bird ranged up to 10 ml. This fluid appeared to be some of the material escaping from regressing ova but of thinner consistency.

The changes in the oviduct which took place in birds with infectious bronchitis depended largely on the phase of infection. During the acute phase of the disease, the oviducts decreased in size to as little as 10.5 grams in weight and 11.5 inches in length. Birds with infectious bronchitis were under physiological stress because of lowered feed and water consumption. It is not surprising, therefore, that some of the changes were similar to those described in group S (figure 1).

Fig. 1. Reproductive tracts from 1504 a control bird, 1650 an infected bird 9 days after inoculation with bronchitis, and 1654 a bird 9 days after physiological stress. Birds 1650 and 1654 were out of production for 6 days at the time of sacrifice.

Oviducts of decreased weight and length were seen till the 21st day after inoculation.

In an earlier part of this paper, mention was also made of a soft, white, granular material found in the firm part of the albumen of certain eggs. Similar material attached to the albumen-secreting region of the oviduct was found in the birds producing the affected eggs.

When fresh oviducts from infected birds were longitudinally cut and laid out, no difference in appearance of the epithelial surface was observed from those of groups S and C. Upon fixation in formalin, however, about one-sixth of the group I oviducts showed the line of demarcation between the isthmus and uterus more clearly than could be seen in the oviducts of the control birds and the birds placed under stress. In the infected oviducts that had this line of demarcation, the color of epithelial surface of the uterus appeared darker than that of the isthmus. The longitudinal folds, especially of the isthmus, appeared more plump and closer together than those normally found at the junction of the isthmus and uterus (figure 2).

Ovarian changes similar to those described in birds of group S were seen in the infected birds from 5 to 21 days after inoculation (figure 3). All infected birds sacrificed beyond the seventh week after inoculation had ovaries of normal weight and oviducts of normal weight and length.

Within the first 4 or 5 days of the infection, the peritoneal cavity of group I birds contained the same type and amount of fluid as described in birds of group S. From 5 or 6 days to 12 weeks later, 75 percent of the group I birds examined showed a light to moderate amount of yellow, cheesy material within the peritoneal cavity. It was often attached to the peritoneum lining the posterior abdominal wall. Some of this material was sectioned and was found to be similar to shell membranes, egg yolk, and albumen.

THE MICROSCOPIC PATHOLOGY OF THE REPRODUCTIVE TRACT

THE OVARY

Group C (control). Five follicular stages were observed

Fig. 2. Opened formalin fixed oviducts at the isthmo-uterine junction. Left oviduct is from a control bird. Middle oviduct is from an experimentally infected bird 60 days after inoculation (junction is at pin holes). Oviduct on right is from a bird 7 days after being placed under physiological stress.

in the ovaries of the control birds. These were primary follicles, growing follicles, mature follicles, discharged follicles, and atretic follicles. Atretic follicles were seen in the early stages of development, which is considered normal. This kind of atresia was similar to that described for mammals.[13] None of the nearly mature follicles was observed to be atretic. Occasionally small numbers of yolk spheres and eosinophilic homogeneous material were found microscopically among the ovarian tissue elements. Two of the seven birds had a mild granulocytic infiltration (predominantly heterophils) of the ovarian cortex.

Group S (stressed). Physiological stress (food and water deprivation) produced changes in the ovary that were most severe within 5 to 15 days post-stress. In contrast to group C, atresia was more pronounced in the more mature follicles. The degree of change varied from ovary to ovary and from follicle to follicle. Atresia in the early stages of follicular development has been mentioned above under group C.

Atresia in the more mature follicles was marked in the early stages by the presence of red blood cells internal and external to the granulosal membrane and by the rupture of the granulosal membrane and thecae to allow the escape of yolk material. This rupture was almost always toward the side of the follicle attachment. In most instances, the extruding yolk material was contained by the mesothelial tissue surrounding each follicle. The thecal membranes had contracted as if to excrete the yolk material actively. As a result, the thecae on cross section appeared much thickened and shortened. The mesothelium in some areas was very thin. The granulosal membrane that had lined the thecal wall became convoluted and invaginated in fingerlike fashion into the remaining yolk material. In some areas there was granulosal cell proliferation. Heterophilic infiltration of these areas was seen.

In later stages of atresia, the mesothelium and theca externa were thickened and highly vascularized. Capillaries and connective tissue originating from the mesothelium and theca externa permeated the extruded yolk material. Yolk that was still contained within the thecal walls was invaded by granu-

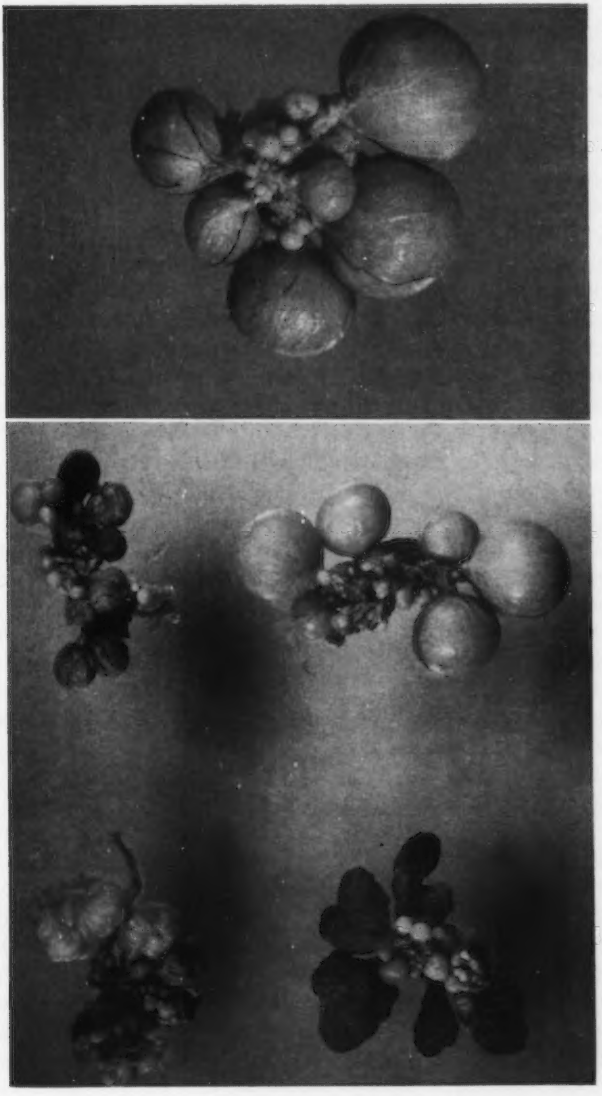

Fig. 3. Ovaries on left from chickens 5 days after physiological stress. Ovaries in center 5 days after infection with bronchitis. Ovary on right from a normal bird.

losal cells, red blood cells, and heterophils.

Considerable amounts of yolk spheres were seen within the ovarian cortex and medulla. Most of this material came via the yolk stalk from the atretic follicles already described. The cortex was infiltrated by heterophils.

Group I (infected). The ovarian changes in the infected birds were similar to those described under group S, but were milder in degree and extended over a longer period of time. As in group S, the more mature follicles showed the greater amount of change. However, fewer follicles (percentage) at any given time became atretic. Atresia was seen in older follicles up to 60 days postinfection. The theca externa and mesothelium in about a quarter of the maturing follicles examined from birds sacrificed during the later phase of the experiment, were irregular in their thicknesses within a given follicle. In some instances, these two membranes were separated by a pink homogeneous material and red blood cells.

EPITHELIAL LINING OF THE OVIDUCT

Group C. The epithelial lining of the oviduct of control birds had a normal microscopic appearance (figure 4). Height measurements of the epithelial cells and the various portions of the oviduct had the following ranges during the time when group C birds were being sacrificed.

Posterior funnel	18.6 to 28.3 microns
Middle albumen-secreting regions	12.9 to 20.4 microns
Posterior isthmus	16.8 to 29.1 microns
Posterior uterus	20.4 to 27.9 microns

Group S. The epithelium of the posterior funnel was lower than normal starting on the 5th day after the beginning of stress. On the 7th day it decreased to as low as 10.71 microns. The epithelial cells, however, did not show a change in shape because the atrophy of the elements making up the oviduct appeared to be proportional. There was no significant loss of cilia. Decrease in the height of the epithelial lining of other portions of the oviduct was not significant. When birds of group S were fed their normal ration, return to the normal height of all the cells of the epithelial lining took place in another 10 days.

Group I. The cells of the epithelial lining of the oviduct of birds in group I decreased in height and took on cuboidal shapes during the period when the disease was most active. This was especially true of the goblet cells. The decrease in height of the epithelial cells was not uniform in the various parts of the same oviduct. The cilia, which normally cover the surface of the epithelium, decreased in numbers and in many places were entirely absent (figure 5).

The decreases in epithelial height, which took place at various levels of the oviduct, ocurred primarily between the 7th and 21st days of infection. The epithelium of a posterior funnel was abnormally decreased in three of six birds sacrificed during this period. It was as low as 9.85 microns in one bird sacrificed on the 7th day of infection. The epithelium of the middle albumen secreting region was abnormally low in five of the six birds. Two birds sacrificed on the 9th and 13th days of infection had epithelium as low as 6.21 microns. Five of the six birds had abnormally low epithelia in both posterior isthmus and posterior uterus. The epithelial height of the posterior isthmus decreased to a low of 3.85 microns in one bird sacrificed on the 7th day of infection. On the 9th day of infection, the epithelial height of the posterior uterus had decreased to 5.78 microns in another bird.

Recovery was slow, for no oviduct from group I birds examined between the 7th and 21st days of infection had a normal height of epithelium at all levels of the oviduct. With three exceptions, 15 oviducts examined subsequent to the 7th week of infection had epithelial cells of a normal height compared to controls. Although sparse on an occasional surface area, cilia in general had also returned to a normal appearance.

TUBULAR GLANDS OF THE OVIDUCT

Group C. Widely scattered dilated glands with lumens ranging from 0.025 to 0.2 micron in diameter were found in only three of seven oviducts. Some of these glands contained within their lumens a small amount of homogeneous material which stained lightly with eosin. The epithelium that lined these glands was cuboidal to flat in outline. A few dilated glands were located in the posterior region of the funnel of one bird and in the albumen-secreting region of the other two birds.

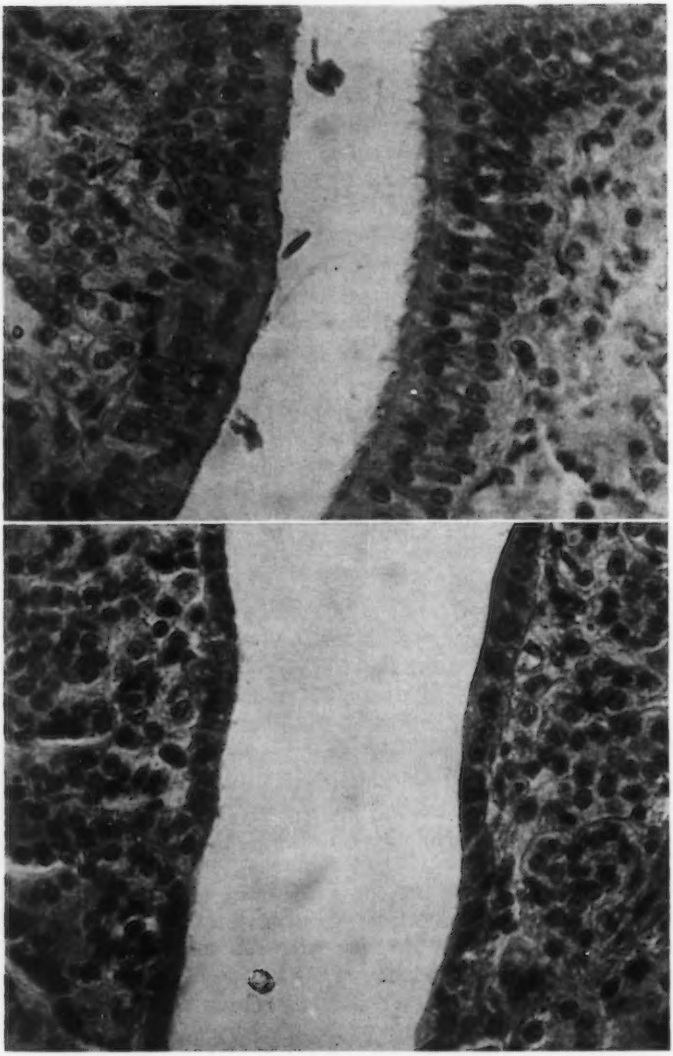

Fig. 4. (top) Epithelial lining of the posterior uterus of the oviduct of a control bird ×760.

Fig. 5. (bottom) Epithelial lining of the posterior uterus of the oviduct of an experimentally infected bird 9 days after inoculation showing cuboidal shaped cells and loss of cilia from the epithelial surface ×760.

Group S. Glandular dilation was found in three of the ten oviducts examined from birds in group S. The degree of dilation of glands was similar to that found in group C, but the distribution involved more parts of the oviduct. The areas involved were the posterior regions of the funnel, the albumen secreting region and the uterus. Certain birds sacrificed immediately after the stress period showed the most involvement. The appearance and the size of the dilated gland were similar to those described under group C.

Group I. Fifteen of the 24 oviducts examined from birds of group I had glandular dilation. The epithelia and contents of the dilated glands were similar to those described under group C. However, the size, incidence, and distribution of these glands in group I were greater than in the other groups (figures 6 and 7). The sizes of these dilated glands measured from 0.03 to 1.0 mm in diameter, though the average was near 0.15 mm. The dilated glands were located from the middle portion of the funnel to the posterior region of the uterus. The incidence was greatest in the albumen secreting regions.

LAMINA-PROPRIA AND INTERTUBULAR STROMA OF THE OVIDUCT

Group C. Of the seven control birds, three that were sacrificed during the last half of the experiment showed lymphocytic foci or diffuse infiltration of inflammatory cells with a small degree of fibroplasia in the lamina-propria and the intertubular stroma of the oviducts. The focal aggregations that were found in only two birds were made up of mature and immature lymphocytes. A diffuse cellular infiltration into the lamina-propria and the intertubular stroma of the oviduct was found in two of the seven birds examined. Plasma cells, mononuclear wandering cells, heterophils and lymphocytes were the chief cells.

Group S. Lymphocytic foci and diffuse cellular infiltration with a small amount of fibroplasia as described under group C were also found in group S. Birds sacrificed within 3 weeks after being placed under stress showed slightly more cells than the control birds. Moreover, some of the lymphocytic aggregates were of sufficient size so that the delimiting membranes were seen. These membranes were made up of

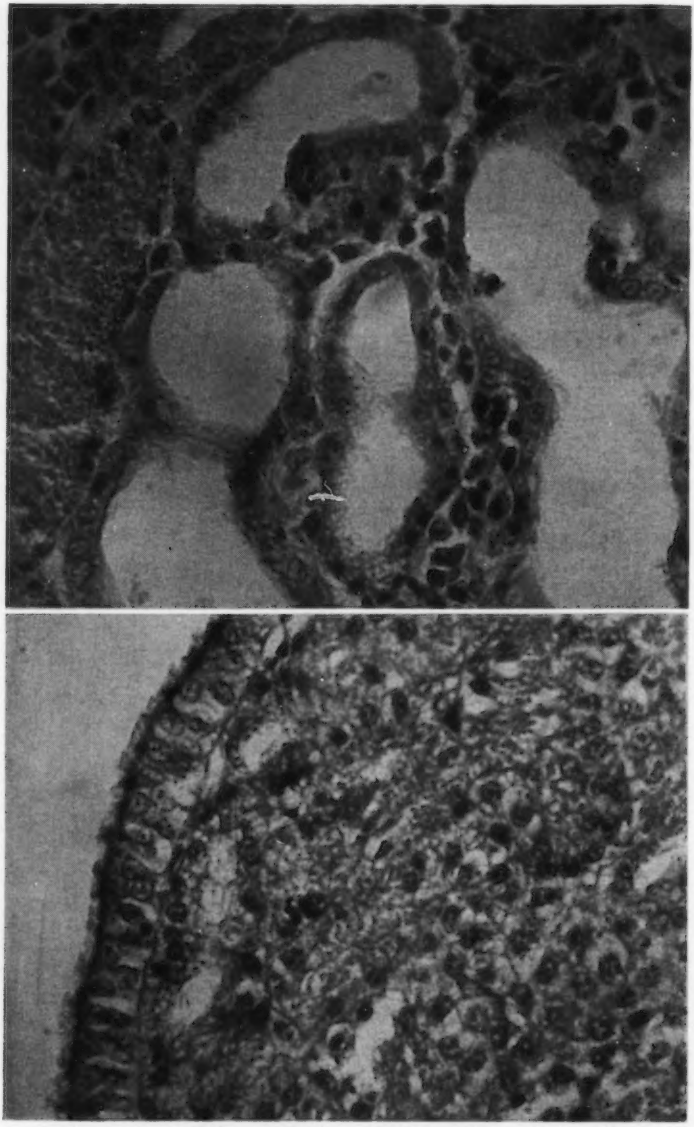

Fig. 6. (top) Dilated tubular glands in the anterior albumen secreting region of the oviduct of an experimentally infected bird 55 days after inoculation ×760.

Fig. 7. (bottom) Tubular glands in the anterior albumen secreting region of the oviduct of a control bird ×760.

intertubular connective tissue that was continually being pushed out by intrafocal cellular multiplication, thereby forming a "pseudomembrane."

Group I. Lymphocytic foci and cellular infiltration in the lamina-propria and the intertubular stroma of the oviducts were most extensive in the birds of group I. The foci of lymphocytic cells reached up to one mm in diameter. Although these nodules of considerable size were in the intertubular connective tissue as well as in the lamina-propria, the glands around them did not appear to be significantly compressed. "Pseudomembranes" were very pronounced in some instances.

The diffuse areas of cellular infiltration were made up primarily of plasma cells, mononuclear cells, and lymphocytes. The number of plasma cells increased more than two-fold in the infected birds over those that were noninfected. Mononuclear cells were present in the oviduct tissue of group I birds in greater numbers from the 6th to 76th day of infection than at other times. Heterophilic infiltration of the lamina-propria and the intertubular connective tissue of the oviduct occurred in the birds of group I most intensively between 8 and 61 days after inoculation.

Varying degrees of fibroplasia and edema were observed in the lamina-propria and the intertubular connective tissue of the entire oviduct in about two-thirds of the infected birds examined. Where fibroplasia was extensive, glandular elements were few in number or entirely absent. The portion of the oviduct that had been most affected by this change was the uterus as shown in figure 8. For comparison, normal tissue is shown in figure 9.

MUSCULAR LAYERS OF THE OVIDUCT

The elements comprising the muscular layers of the oviduct in all three groups showed little significant change other than a diffuse infiltration with the inflammatory cells. The muscular layers of the oviducts from group I birds had a heavier infiltration of the inflammatory cells when compared to the oviducts of the noninfected birds. The types and locations of cells, however, were similar in all three groups.

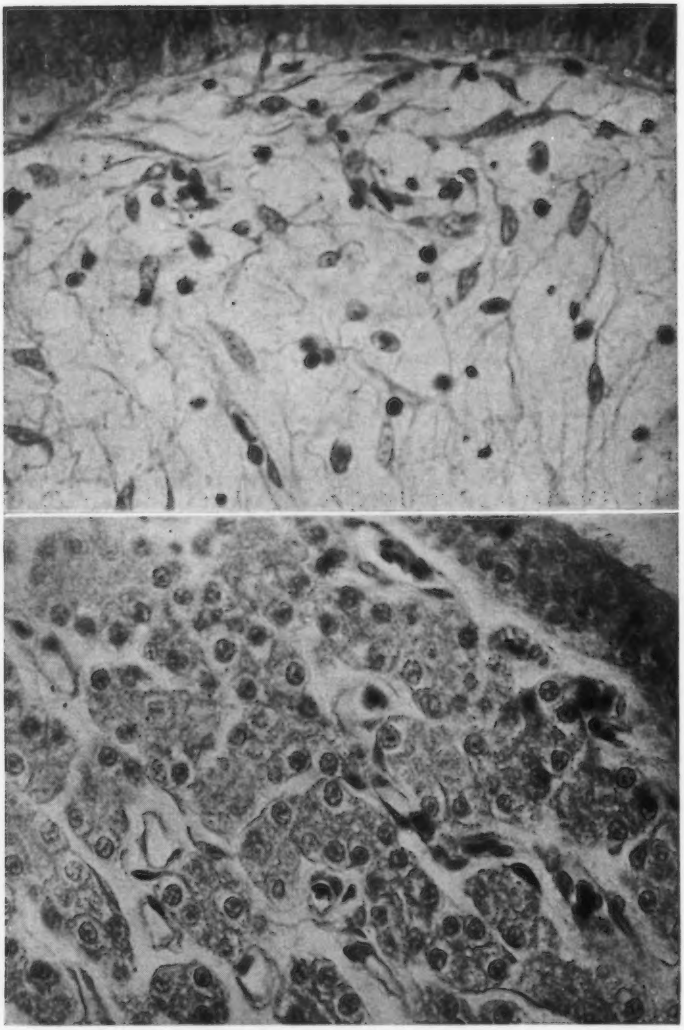

Fig. 8. (top) Presence of fibroblasts and absence of tubular glands in the posterior uterus of the oviduct of an experimentally infected bird 74 days after inoculation ×760.

Fig. 9. (bottom) Tubular glands in the posterior uterus of the oviduct of a control bird ×760.

DISCUSSION

The data on egg production and egg quality bore out similar observations made by Van Roekel and others in field outbreaks. The decreases in production of groups I and S were closely parallel, with group S having declined more rapidly. This is explained by the fact that birds of group I, even at the height of infection, consumed some feed and water, while the birds of group S had been completely deprived. Normal consumption of feed and water by group S birds was established as soon as their rations were restored, whereas the infected birds took more than 3 weeks to return to their former level of feed and water intake. The return to production by group S began within 8 days after the onset of the stress period, but group I did not begin production until 20 days after inoculation. The average number of days that group S birds were out of production was only 11 days, whereas group I birds stayed out of production for an average of 35 days. Group S birds returned to production at a higher rate, but many of the infected birds returned at a low rate. The control birds of group C maintained normal production. Average weights of eggs of group I birds declined nearly 0.5 ounce per dozen during the first 7 weeks of infection. The noninfected groups, however, continued to make gains of more than an ounce per dozen over a comparable period. These young laying pullets normally would be expected to increase their egg size during their 1st year of production. Groups S and C conformed to this pattern, even though the former group had been subjected to a brief period of starvation and thirst. In the infected group, however, some birds were unable to increase their egg weights, and other birds actually decreased in their egg weights. The 1.5 ounce per dozen difference in average egg weights between the infected group and the noninfected groups could mean the difference between profit and loss to many poultrymen.

There was a slight lowering of albumen heights of broken-out eggs from about 22 percent of the noninfected birds over a 7-week period. This is not unusual nor serious in a normal laying flock. However, nearly 40 percent of the infected birds

suffered a more serious decrease in albumen height of broken-out eggs.

White granular masses were found intermittently in the albumen of eggs from approximately one-fifth of the infected birds. These granular masses gave an objectionable appearance to the broken-out egg. Birds producing these eggs also laid eggs with a poor consistency of firm albumen. Shell thickness was significantly reduced in eggs from 37 percent of the infected birds so that the eggs were unmarketable. Some of these thin-shelled eggs, as well as others, were misshapen. Egg shells from 10 percent of the infected birds had white calcareous deposits on the outside surface.

It is well known that birds with infectious bronchitis consume less feed and water than normal. This not only has an effect on egg production but on the reproductive tract as well. For this reason, group S, which was not infected with virus, was subjected to stress conditions. Only by examining the reproductive tracts of the control birds and the birds placed under stress could the data from the infected birds be correctly interpreted. The initial changes that took place in birds with infectious bronchitis appeared to be largely the effect of decreased feed and water consumption. However, the reproductive tracts of the infected birds reutrned to normal sizes and weights more slowly than those of group S birds.

Ovarian atresia was found in both groups I and S. This retrogressive process was a result of stress conditions, caused by a decrease in feed and water consumption or disease, or both. Greater stress resulted in more pronounced and rapid atresia. Slow return to normal feed consumption after infection by group I birds may be related to atresia in maturing follicles found in the infected birds over longer periods of time than in group S birds. This, in part, may have accounted for a slow return to egg production.

Cole[3] reported that oviducts from birds that produced eggs with firm albumen had higher goblet cells in the albumen secreting region than did oviducts from birds that produced eggs with poor consistency of firm albumen. He deducted that the higher goblet cell produced more mucin; thus a de-

crease in the height of the albumen in eggs should be expected when the height of the goblet cell is reduced. Six infected birds were sacrificed between the 7th and 21st days after infection. In five of these birds the epithelium in the middle albumen secreting region had decreased in height as compared to controls, but no significant lowering in the height of firm albumen occurred in eggs from group I during this time nor during the following 2 weeks. On the other hand, five birds sacrificed after the 7th week of infection had a normal height of epithelium yet had produced eggs with poor albumen consistency. Therefore, our data did not support any correlation between firm albumen and goblet cell height.

Available data on frequency, size, and distribution of dilated glands in the oviduct did not support any relationship with egg quality and egg size. It appeared that the frequency, size, and distribution of dilated glands in the oviduct increased directly with the degree and duration of stress conditions.

Inflammatory cells, with focal and diffuse distributions, were found much more often in the oviducts of bronchitis infected birds than in those of noninfected birds. Also, focal aggregations of mature and immature lymphocytes were much larger in the infected oviducts. Most of these lymphocytic lesions were found in glandular layers. The proliferation of lymphocytic cells in many of the foci pushed out the surrounding intertubular stroma to form delimiting membranes. The lymphocytic foci did not have any structural supporting tissues of their own as commonly seen in the mammalian nodes. Biswal[1] in his report on 19 healthy control birds, described these foci in the oviduct as lacking in delimiting membranes. Lucas,[12] who had found similar lesions in the liver, pancreas, and spleen, indicated a close association between the number of these focal areas and the predisposition toward the development of lymphocytomas at a later date. Two birds of the infected group died of lymphomatosis, but none of the noninfected birds died from this cause.

The diffuse inflammatory cells in the oviducts of group I birds consisted of mature lymphocytes, heterophils, mononuclear wandering cells, and plasma cells. Jungherr[10] described these changes together with the lymphocytic foci as sug-

gestive of chronic inflammation and probably having nothing to do with lymphomatosis. The concentration of cells in diffuse inflammation was greater in the glandular layer than in other areas of the oviduct. Thus, the concentration and distribution of focal and diffuse inflammatory cells indicated that the glandular layer of the oviduct was the area in which the most extensive and severe inflammatory changes took place within the oviducts of birds with infectious bronchitis.

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SUMMARY

The effects of infectious bronchitis on the reproductive system of White Leghorn hens were studied. One hundred twenty-six susceptible hens were divided into three matched groups. Group I was infected with bronchitis. Group S was not infected but subjected to the temporary stress of feed and water deprivation to approximate the effects of a decline in feed and water intake that accompanies bronchitis infection. Group C was the non-infected and non-stressed control.

1. Egg production in group I dropped almost to zero by the 10th day post-inoculation. Up to five weeks after infection, only 30 percent of the birds came back to production. The average time out of production was 35 days. Group S ceased egg production in 4 days but the average time out of production was only 11 days.

2. Egg weights of group I dropped 0.46 ounces per dozen at 7 weeks post inoculation. Egg weights of groups S and C increased 1.76 and 1.06 ounces per dozen respectively.

3. Internal egg quality (U.S.D.A. score units) in group I 7 weeks after infection decreased from three to five units in 39 percent of the birds. There was no comparable decrease in egg quality in groups S and C.

4. White granular masses in the albumen were found in eggs laid by 20 percent of the group I birds. None were ob-

served in the albumen of the other two groups.

5. Misshapen eggs were produced by one-sixth of group I birds from 5 to 50 percent of the time. No misshapen eggs were laid by birds in the other two groups.

6. Soft-shelled eggs were laid consistently by 25 percent of group I birds. Six percent laid them intermittently. Thin-shelled eggs were produced by 12 percent 7 weeks after infection. No abnormalities in egg shell thickness appeared in the other two groups.

7. Egg shells from 10 percent of group I birds had verrucous, calcareous deposits on the outside surfaces. Shell surfaces were normal in the other two groups.

8. Oviduct length and weight of birds in groups I and S were markedly reduced during the acute bronchitis infection (group I) and stress (group S) respectively. Normal size was regained in 11 days by group S but not until after 21 days in group I.

9. Ovarian regression was marked in group I to 21 days and group S to 15 days after inoculation and stress respectively. Leakage of yolk into the body cavity was common.

10. A sharp line of separation between the isthmus and uterus in formalin-fixed oviducts could be seen in one-sixth of the birds of group I. This anatomical division was not as obvious in the other groups.

11. Microscopic changes of the atretic follicles from groups I and S consisted of rupture of the thecae and granulosal membrane, release of yolk, and convolution and invagination of the membrane into the remaining yolk. Later, thickening and vascularization of the mesothelium and theca externa occurred.

12. The height of the epithelial-lining cells of the oviduct was sharply reduced at various levels in group I and the cells took on a cuboidal shape, especially the goblet cells. There was a decrease in number of cilia and in places a complete absence of cilia.

In group S there was a diminution in size of epithelial cells but no distortion of shape. The cilia were not effected.

13. Dilation of glands occurred in more than half the ovi-

ducts examined from group I. The frequency and severity of the dilation were significantly greater than in group S.

14. In the lamina propria and intertubular stroma, numerous lymphocytic foci and extensive cellular infiltration were present in the oviducts of group I. Lymphocytes, plasma and mononuclear cells and heterophils comprised the infiltrating cells. Fibroplasia and edema in these areas were present in varying degrees in two-thirds of the birds. Significantly fewer lesions were seen in group S and least in group C.

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EGG TRANSMISSION OF INFECTIOUS SINUSITIS OF TURKEYS

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IT IS generally accepted that egg transmission is an important means of spread of infectious sinusitis of turkeys and of chronic respiratory disease of chickens—two diseases apparently caused by the same organism, *Mycoplasma gallinarum*. Jersted *et al.*[5] obtained evidence of egg transmission of turkey sinusitis when they isolated the sinusitis agent from two poults from pipped eggs. Van Roekel *et al.*[7] were able to isolate *M. gallinarum* from embryos and chicks originating from naturally infected chicken breeding flocks. Further evidence of egg transmission of these diseases has been obtained by others.[1,2,3,6]

The purpose of this report is to furnish additional evidence supporting egg transmission as an important factor in the spread of infectious sinusitis of turkeys.

PROCEDURE

The first source of eggs and poults was a hatchery that was having outbreaks of infectious sinusitis in three-week-old poults. This hatchery supplied us with eggs which had failed to hatch and culled day-old poults originating from its 31 breeder flocks. Most of the eggs received had been pipped. The second source of eggs was a flock of turkeys that was going through an outbreak of infectious sinusitis. These eggs were incubated and hatched in isolation at the laboratory.

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† Technical assistance by Francais Weddle.

The dead poults removed from eggs which failed to hatch, were examined for evidence of air sac infection. If lesions were found, the air sac or exudate was removed and cultured in chicken or turkey meat infusion broth.[4] Live poults were similarly examined. If air sac lesions were not found, cultures were not prepared. Tissue pools were usually saved when several poults in the same lot had lesions. The infected air sacs with exudate were ground, suspended in broth and held at —25 C until the results of the initial cultures were available. When cultures were negative, the tissue pools were used to check further for the presence of *M. gallinarum* by inoculation of turkeys and by inoculation of deembryonated eggs.[4] Tissue pools were saved from the first source of live, culled poults with air sac lesions as follows: one each from flocks 2, 8, 13, 21, 22 and 26; two pools each from flocks 14 and 30; three pools each from flocks 12 and 15.

Turkeys used for inoculation were bronze poults from 3 weeks to 3 months of age. They were obtained from a local hatchery as day-old poults and raised on wire in an isolation room. Prior to inoculation they were placed in individual cages or batteries. The turkeys were usually inoculated into one sinus and into one of the posterior thoracic air sacs. After inoculation, they were observed for two or three weeks, killed and examined for sinus and air sac lesions. Cultures were made only if sinus exudate or air sac thickening was evident.

M. gallinarum was identified by its initial granular growth in broth, its ability to grow in the presence of thallous acetate and penicillin, its typical colonial form on avian serum agar, its clumped, coccoidlike form observed in preparations stained with Giemsa's stain, and its ability to produce sinusitis or air sac infection in turkeys.

RESULTS

The results of the studies made on the eggs and poults originating from 31 breeding flocks are presented in table I. Eggs from 18 of these flocks were found to produce poults with air sac lesions. The incidence of air sac lesions in the poults dead in the egg was lower (2.6 percent) than found in the live, culled, day-old poults (6.9 percent).

TABLE I

Results of examinations and cultures of poults from eggs and live, culled, day-old poults from the same sources.

Flock	Poults taken from eggs	Air sac lesions	Cultures	Day-old culled poults	Air sac lesions	Cultures
1	76	2	—*			
2	109	6	3+**	164	11	—
3	27	1	—	22	2	—
4	10	0				
5	84	0		51	0	—
6	25	1	—	54	3	—
7	65	1	—	144	6	—
8	47	3	2+	91	3	—
9	28	0		53	0	
10	41	0				
11	4	0				
12	41	4	2+	181	14	—
13	24	1	—	37	2	—
14	68	1	1+	154	21	—
15	75	4	3+	205	17	—
16	24	0				
17	74	0		119	12	—
18	6	0				
19	14	0		27	0	
20	9	0		43	0	
21	12	0		62	2	—
22	60	0		101	11	—
23	5	0		26	0	
24	5	0				
25	17	0		26	1	—
26	2	0		33	2	—
27	7	1	1+	10	0	
28	10	0		18	0	
29	3	0		16	0	
30	36	2	—	96	13	—
31				8	1	—

* Negative. **Cultures from 3 different poults positive for PPLO.

A total of 12 isolations of *M. gallinarum* was obtained from poults dead in the egg. No isolations were obtained from live, day-old poults. The tissue pools from the latter, checked for *M. gallinarum* by inoculation into turkeys and in deembryonated eggs were also negative.

The results obtained with the eggs received from the second source are summarized in table II. Two hundred eggs

TABLE II

Results of examining and culturing embryos and poults from second source

Number of embryos or poults	Age in days	Number having air sac lesions	Culture *M. gallinarum* isolated
2(emb.)		2*	2+†
22 pipped and failed to hatch	0	6	5+
12 culls and cripples	1	6§	1+
25	1	10	—
25	2	10	—
1 dying	5	1	—
1 dying	9	1	—
7	13	5	—
1 sinusitis	17	1	1+
8 sinusitis and rales	18	7	2+††
1 died	19	1	1+
2	46	2	2+

* Both embryos had lesions. † Isolation obtained from both embryos. § Inoculated poults developed air sac lesions yielding *M. gallinarum*. †† One isolation from lung of poult with no gross lesions.

were set. They were candled on days 16, 18, 23 and 25. A total of 20 dead embryos were removed in addition to the infertile eggs removed on the first candling. After one poult developed sinusitis on the 17th day other poults had swollen sinuses and tracheal rales on the next day. Many poults had respiratory symptoms on day 24. Signs continued in the poults and the remaining 38 birds were used in an experiment to be reported in the following paper.

DISCUSSION

The thoracic air sacs were most often involved. They were thickened and contained caseous exudate. It was uncommon to find the abdominal air sacs involved. The presence of infected air sacs in the poults prior to and shortly after hatching and the isolation of *M. gallinarum* from some of them indicates that *M. gallinarum* was present in the egg at the time it was laid.

The outbreak of sinusitis in the poults hatched from the second group of eggs is additional evidence that infectious

sinusitis can be transmitted through the egg. This outbreak had been anticipated because of the high incidence of air sac lesions (40 percent) in the hatched poults. These poults were in strict isolation and the infection was not likely to have come from a source other than the poults themselves. The only abnormal condition to which the poults were subjected was a moderate chilling due to brooder failure at one week of age. This chilling may have been a factor in precipitating the outbreak.

With the exception of a single isolation from one of the six culled or crippled poults at 1 day of age (table II) it was not possible to isolate *M. gallinarum* from live poults even though many more attempts were made. The organism was isolated however from poults dead in the shell. This was particularly obvious with material from the second source of eggs where a number of isolations were obtained from poults prior to hatching and after the outbreak started. However, from 20 poults with lesions at 1 and 2 days of age, not a single isolate was obtained.

This failure to isolate the organism from live poults might be explained on the basis of passive antibody interference. However, attempts to neutralize the organism in vitro with immune serum were not very successful. Certain strains adapted to grow in broth will grow in medium containing 20 percent avian serum having a high agglutination titer against *M. gallinarum*. The fact that a total of 26 isolations were made during this work indicates that the medium used was satisfactory.

SUMMARY

Evidence is presented that infectious sinusitis of turkeys is transmitted through the egg. Culled poults and eggs which failed to hatch were obtained from one hatchery which was experiencing outbreaks of sinusitis in 3-week-old poults. These were examined for air sac lesions and cultured for *Mycoplasma gallinarum*. Twelve isolations were made from six different supply flocks. While isolations were made from poults removed from the shell, it was not possible to isolate the organ-

ism from live, culled poults. In another experiment, fresh, fertile eggs were obtained from a flock suffering from infectious sinusitis. The eggs were incubated and hatched in isolation. The organism was isolated from dead embryos and poults dead in the shell, but with one exception, no isolations were made from live poults even though they had air sac lesions. Poults from these eggs raised in batteries suffered an outbreak of sinusitis between 2 and 3 weeks of age. This indicates that *M. gallinarum* infection may be transmitted to poults by way of the egg.

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A SEROLOGICAL STUDY OF INFECTIOUS SINUSITIS IN TURKEYS

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SEROLOGICAL testing of turkeys to detect carriers of infectious sinusitis may be useful in the future control of

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the disease. Jungherr *et al.*[6] demonstrated the application of the hemagglutination-inhibition (HI) test in detecting pleuropneumonia-like infection in chickens. Fahey[4] applied the HI test to testing of turkeys for infectious sinusitis. He used a turkey isolate of *Mycoplasma gallinarum* as antigen. Adler[1] outlined a procedure for a rapid, slide agglutination test for the diagnosis of chronic respiratory disease in chickens and infectious sinusitis in turkeys. The diagnosis of these conditions by serological procedures has been carried on rather extensively by Jungherr *et al.*,[7] Crawley and Fahey,[2] and Dunlop.[3]

The purpose of this study was to determine the agglutination and HI titers present in a group of naturally infected turkeys for a period of 12 to 21 months.

MATERIALS AND METHODS

The origin of the major group of 38 turkeys used in this work was reported in the preceding paper. This group of poults suffered an egg-borne outbreak of infectious sinusitis when 3 weeks of age (March 12, 1955). Some of the birds in this group were treated with antibiotics beginning May 10, 1955 (group 2). The untreated birds were placed in group 1. Group 3 obtained as day-old poults April 1, 1955, from a different source and raised in isolation, was introduced into the infected flock June 6, 1955 while many poults in group 1 and 2 still had swollen sinuses and tracheal rales. Group 4 (five birds) originating from the same hatchery as group 3 was introduced into the flock in January 1956. Groups 1 and 2 were raised in batteries until June 6, 1955, when they were moved to an open shed with a wire floor during the summer. In October they were moved to a heated barn and placed on litter. They were divided among four stalls, and were grouped according to their serum titers irrespective of the original groups. The reason for such grouping was to collect eggs from each pen of birds for hatching; however, since fertile eggs were not obtained this phase of the experiment had to be abandoned.

Beginning in May of 1955 and continuing through December of 1956 the birds were bled at irregular intervals for

serological testing by the tube agglutination test, the serum plate agglutination test, and the HI test. The serum plate test was run on all the samples through the April 6, 1956 bleeding and then discontinued because of the difficulty in reading the test and poor correlation with the other two serological procedures. The serum plate test results are not recorded.

The antigen for agglutination tests was prepared according to previously described procedure.[5] The strain used was 5969, a Massachusetts strain obtained from the University of Connecticut. For the antigen, the unstained plate antigen with a density of two times the no. 10 tube of the McFarland nephelometer scale, was diluted 1:15. The antigen used in the HI tests was either 5969 or 595 depending on which had the better titer at the time tests were conducted. Strain 595-Iowa is an isolate from turkeys. Initially, antigen refrigerated only a few days was used, but later, frozen and lyophilized antigen was used in the HI tests.

The tube agglutination test was carried out with .08, .04, .02 or .01 ml serum to which was added 1 ml antigen resulting in dilutions of 1:12.5, 1:25, 1:50 and 1:100 respectively. Tubes 12 by 100 mm were used. The tests were incubated at 37 C and read in 18 to 24 hours. Clearing of the supernatant fluid was the main criterion used in reading the agglutination tests. The tests were read as either positive or negative.

The serum plate test was performed by mixing equal parts of serum and plate antigen on a glass plate and observing the tests for 3 to 4 minutes. The antigen was also used with equal parts of 20 percent NaCl solution in an attempt to hasten the reaction. In this case the amount of antigen was doubled.

In the HI test 12 by 100 mm tubes with rounded bottoms were used. The hemagglutination (HA) titer of the concentrated antigen was first determined by making an initial 1:50 dilution of antigen in saline followed by two-fold (0.5 ml + 0.5 ml) dilutions through 1:1600. Another series of antigen dilutions was made by making an initial 1:75 dilution followed by two-fold dilutions through 1:2400. To these antigen dilu-

tions, 0.5 ml of 0.25 percent washed, turkey, red blood cells were added making a total of 1 ml in each tube. A control tube consisted of 0.5 ml cells and 0.5 ml saline. After allowing the tests to set about 1 hour at room temperature, the tubes were examined for agglutination of the cells. Sliding of the cells in the rounded bottom of the tube as the tubes were tilted was the criterion used in determining agglutination. If agglutination had taken place, the cells did not slide whereas in the negative test the cells began to slide as the tubes were tilted.

In the HI procedure, two HA units were used in the test. The serum dilutions were initiated by making a 1:5 dilution (0.2 ml serum + 0.8 ml saline). This became the serum control tube in the test. The serum dilutions were continued by doubling the dilutions directly in 0.5 ml amounts of the antigen. Washed turkey red cells (0.25 percent were added in 0.5 ml amounts. The tests were read after 1 hour or more at room temperature.

RESULTS

The results of the tube agglutination and HI tests in group 1 turkeys (natural outbreak March 1955) are summarized in table I and II respectively. In general the agglutination titer declined as clinical signs disappeared, although many of the birds remained positive (1:12.5 or higher) 15 months after infection. The decrease in the number of birds tested on September 14, 1956, was due to killing of the toms. Only one of the birds that was positive to the agglutination test at the September 14, 1956 bleeding and thereafter had visible sinusitis. However three others were found to have a definite sinusitis at necropsy. These four birds yielded *Mycoplasma gallinarum* on cultures. The other two reactor birds had no sinusitis or air sac lesions at necropsy and cultures of lung material were negative for *M. gallinarum*.

The HI test results (table II) revealed that the HI titer was variable, but in general the mean titer did not decrease as rapidly as the agglutination titer. If one considers a 1:20 or above as positive, it can be seen that there were very few turkeys whose sera became negative to the test. The five

TABLE I

Results of tube agglutination tests on serum from group 1 turkeys infected with infectious sinusitis—natural outbreak in poults on March 12, 1955

Bleeding date	Number positive at highest dilution: Serum dilutions neg.	1:12.5	1:25	1:50	1:100	1:200	Index average	Number birds with rales or sinusitis
5/14/55				17*			1:50	21
7/20/55		1	2	6*			1:38.7	11
11/ 5/55	2	3	6	12			1:30.5	6
12/23/55	4	3	4	4	6	2	1:34.7	4
2/15/56	3	2	6	7	4	1	1:35.7	3
4/ 7/56	6	8	4	3	1	1	1:18.5	3
6/14/56	4	8	4	1	2		1:17.7	2
9/14/56	5	1	3	2			1:14.7	1
11/10/56	4	3	2	1			1:12.5	2†
12/19/56	4	2	2	1			1:12.5	3§

* Highest dilution run on random samples. † One, 843, found to have sinusitis at necropsy *(M. gallinarum)*. § Two, 822 and 811, found to have sinusitis at necropsy *(M. gallinarum* isolated from both.) One, 804, had swollen sinus throughout observation period *(M. gallinarum* isolated).

TABLE II

Results of hemagglutination-inhibition tests on serum from group 1 turkeys infected with infectious sinusitis (natural outbreak March 12, 1955).

Bleeding date	Number positive (inhibition) at highest dilution of serum: Serum dilutions neg.	1:10	1:20	1:40	1:80	1:160	1:320	Log. of Geom. mean (HI units)*
7/20/55		1	1	1	3	3	1	149
11/ 5/55		2	4	6	5	5	1	107
12/23/55	1	2	2	4	5	7	2	117
2/15/56	c		3	11	2	3	3	133
4/ 7/56	c	1	5	8	7	2		90
6/14/56	c		5	7	2	3	2	110
9/14/56	c		1	5	1	3	1	140
11/10/56	c			4	3	3		148
12/19/56	c		4	2	1	2		86

* Hemagglutination-inhibition unit = dilution factor of serum x 2 HA units of antigen used in the test. c — Controls negative.

control birds that were introduced in January 1956 were tested at each bleeding and they were always negative even at the 1:10 dilution, or the lowest dilution in the test.

The results of the agglutination and HI tests for group 2 turkeys are presented in tables III and IV, respectively. This group of turkeys, mates of group 1, were treated with antibiotics. A comparison of the agglutination titers with group 1 untreated turkeys reveals that the titers decreased more rapidly in the treated birds although, as in group 1, some remained positive. One bird continued to have visible sinusitis for a year after treatment was discontinued. Sinusitis disappeared after treatment in seven of the nine birds whereas

TABLE III

Results of tube agglutination tests on serum from group 2 turkeys affected with sinusitis and treated with streptomycin and oxytetracycline (terramycin in oil).

Bleeding date	Number positive at highest dilution: Serum dilutions neg.	1:12.5	1:25	1:50	1:100	Index average	Date of Treatment	Number birds with sinusitis
5/14/55				7		1:50	5/10/55[1]	9
7/20/55		1	3	1		1:25	5/17/55[2]	2
11/ 5/55	1	2	5	1		1:20.7	6/ 2/55[3]	2
12/23/55	3	2	1	2	1	1:19.3	7/ 2/55[4]	2
2/15/56	2	2	2	2		1:18.7		2(833)*
4/ 7/56	3	3	1	1		1:12.5		1
6/14/56	3	3	1	1		1:12.5		1(801)*
9/14/56	3	1				1: 3.1		
11/10/56	3	1				1: 3.1		
12/19/56	2	1				1: 4.1		

[1] Each of the 9 birds received 5 ml of terramycin in oil in neck. Five most severely involved with sinusitis received 75 mg streptomycin in affected sinus.

[2] Each received same dose of terramycin. Five birds with sinusitis received from 35 to 150 mg in sinus.

[3] Same dose of terramycin for each; one in addition received 110 mg streptomycin in sinus.

[4] Three birds most severely affected received terramycin in oil.

* Dead. (*M. gallinarum* isolated from sinus exudate).

for the same period in the untreated group of birds, sinusitis or rales disappeared in ten of the 21 birds with clinical signs. The HI results (table IV) reveal a considerably lower mean titer for all the bleedings than in group 1 birds, although most of them remained positive at 1:20 or higher.

The results of group 3 birds (normal birds introduced into infected flock) are summarized in tables V and VI. Most of the turkeys in this group did not develop high agglutination titers. The HI titers were also low. No sinusitis was detected in the group although rales were detected in four birds. It appeared that under the conditions of the experiment there was low spreading ability of the infectious sinusitis agent. Serum samples taken from six mates to group 3 turkeys at the time they were introduced were negative to the agglutination test. A number of birds from this hatch had been used as experimental birds in identification of *M. gallinarum* and had proved susceptible.

TABLE IV

Results of hemagglutination-inhibition tests on serum from group 2 turkeys affected with sinusitis and treated with streptomycin and oxytetracycline (terramycin in oil).

Bleeding date	Number positive (inhibition) at highest dilution of serum							Log. of Geom. mean HI units
	Serum dilutions							
	neg.	1:10	1:20	1:40	1:80	1:160	1:320	
7/20/55		1	3		1		1	71
11/ 5/55		1	3	3	1	1		68
12/23/55	1	2	1	2	1	2		49
2/15/56			4	1	1		1	79
4/ 7/56		1	3	3		1		61
6/14/56		1	3	3				61
9/14/56		2		2				40
11/10/56		1	1	2				47
12/19/56		1	1	1				40

TABLE V

Results of tube agglutination tests on serum samples from group 3 turkeys introduced into the infected flock June 6, 1955.

Bleeding date	Number positive at highest dilutions: Serum dilutions neg.	1:12.5	1:25	1:50	Index average	Number of birds with rales or sinusitis
7/20/55	3			1	1: 9.4	2* rales
11/ 5/55	4	3	2		1: 9.6	none
12/23/55	3	3	1	1	1:12.5	none
2/15/56	2	1	6		1:18.0	none
4/ 7/56	3	4	1	1	1:12.5	none
6/14/56	3	3			1: 6.3	none
9/14/56	5	1			1: 2.0	none
11/10/56	5				0	none
12/19/56	3				0	none

* Rales detected in three birds on 6/15, in 2 birds on 7/2, total of 4.

TABLE VI

Results of hemagglutination-inhibition tests on serum from group 3 turkeys introduced into infected flock on June 6, 1955.

Bleeding date	Number positive (inhibition) at highest dilution of serum: Serum dilutions Neg.	1:10	1:20	1:40	1:80	Log. of Geom. mean HI units
7/20/55	1	1			1	14
11/ 5/55	5	2	2			4
12/23/55	3		3	1	1	13
2/15/56	1	1	2	5		36
4/ 7/56	2		3	4		23
6/14/56	1		4	1		24
9/14/56		1	2	3		50
11/10/56		1	2	2		46
12/19/56			3			40

DISCUSSION

The rapid serum plate method of detecting antibodies to *M. gallinarum* in chicken serum was not directly applicable for turkey serum. Chicken serum containing antibodies to *M. gallinarum* reacts rapidly to plate antigen. A well defined reaction is obtained with samples which are positive at the 1:12.5 dilution in the tube test. With turkey serum, however, the reaction is slow and difficult to read. It is possible that the technic of using one drop of each material was not optimum for the reaction. Pomeroy[8] has suggested using three parts of antigen, one part of serum and up to 5 minutes reading time to make the test more reliable.

The HI test was considered a more sensitive test for antibodies than the tube agglutination test because HI antibodies persisted after the tube agglutination test was negative. The question arises as to what constitutes a positive HI test. None of many samples of serum from normal turkeys was ever positive (inhibition) even at the 1:10 dilution. Thus it may be assumed that a serum causing inhibition in the 1:10 dilution and particularly in the 1:20 dilution or higher is evidence of past or present infection. Attempts to isolate *M. gallinarum* from the low titer birds were negative. *M. gallinarum* was isolated only from birds having lesions of sinusitis. Such birds also had high HI titers. While some of the birds in group 1 became negative to the tube agglutination test, they were positive to the HI test. For example, four of the nine birds at the last bleeding were negative on the tube agglutination test but all had HI titers of 1:20 or higher.

The tube agglutination test is easier to adapt to large scale use in testing turkey flocks because it can be set up like the pullorum and paratyphoid tests. The HI test is more difficult to handle because of the necessity of titrating the antigen and the requirement of washed red blood cells.

The results in group 3 birds suggest that infectious sinusitis was not readily transmitted in semimature and adult turkeys during the summer months or in a heated barn. The five control birds placed in the barn in January 1956 were not in direct contact with the high titer birds but were in

an open-fronted stall across the passageway from the high titer birds. These findings suggest that a portion of a flock may remain susceptible over a long period of time even though *M. gallinarum* is present in the flock.

SUMMARY

Three groups of turkeys with infectious sinusitis were studied serologically for a period of 12 to 21 months. Turkeys which had experienced a natural egg-borne outbreak of infectious sinusitis at 3 weeks of age, infected turkeys treated with antibiotics, and normal turkeys placed with infected ones were studied. The results obtained by the tube agglutination and hemagglutination-inhibition (HI) tests were compared. The rapid serum plate test was also tried but the results were not considered reliable. The tube agglutination tests indicated a drop in titer as the clinical signs disappeared in the birds that had experienced the egg-borne outbreak. While some birds became negative to the tube agglutination test, very few gave negative HI readings. It appeared that the HI test was the more sensitive test for detecting past infections. In infected birds treated with antibiotics over a period of 6 weeks, there was a definite decrease in both the agglutination and HI titer, but some of the birds remained positive. Two of nine birds treated evidenced sinusitis for 8 and 12 months after treatment. Normal birds placed in contact with infected turkeys failed to develop sinusitis, but four developed tracheal rales. The average serum antibody titers in these birds remained low indicating the low virulence of *Mycoplasma gallinarum* under the conditions of the experiment.

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THE EFFECT OF FURAZOLIDONE ON THE CULTURAL AND SEROLOGICAL RESPONSE OF *SALMONELLA TYPHI-MURIUM* INFECTED CHICKENS*

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FURAZOLIDONE (N-5-nitro-2 furfurylidene-3-amino-2 oxazolidone) was shown by Yurchenco, Yurchenco and Piepoli[14] to have activity against Gram-negative bacteria. Smith[9] observed that furazolidone activity against *Salmonella pullorum*, both *in vitro* and *in vivo*, was superior to that of chloramphenicol and sulfamerazine. Furazolidone has been found effective in either reducing or preventing mortality due to *Salmonella gallinarum*[1,4,8] *Salmonella pullorum*[9,12] and *Salmonella typhi-murium*.[10] The workers in the British Isles use a feed level of 0.04 percent for 7 to 14 days which not only prevents mortality but can arrest fecal excretion.[9,11] The practice in the United States is to use a 0.005 percent level continuously for "prevention" and a 0.01 percent level for "treatment" of *Salmonella* infections.

Salmonella typhi-murium accounts for approximately 70 percent of the paratyphoid infections of chickens and turkeys in Virginia.[2] A voluntary testing program was instituted in an attempt to control the disease in breeding flocks. Since many hatcheries maintain their breeding flocks on furazolidone medicated feed, it was felt necessary to determine the

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effect of simultaneous medication and infection on the carrier and reactor states.

MATERIALS AND METHODS

In the preliminary experiment 100 cross bred chicks were used. Each regime was housed in modified Horsfall Bauer isolation cages heated with thermostatically controlled electric light bulbs. For the rest of the study, White Leghorn chickens were raised in wire floored batteries until they were 10 weeks or 10 months of age, and then placed in groups of two or three chickens in modified Horsfall Bauer isolation cages for the experiment. Daily cleaning of cages, housing of small groups of birds, and isolation of each experimental regime were practiced to minimize cross infection and reinfection. *Salmonella typhi-murium* CDC 2606/55, a chicken isolate which has an LD_{50} of $10^{3.5}$ cells for day old chicks, was used throughout the study. After fasting for 18 hours, each chicken was inoculated by pipetting 1 ml of a McFarland No. 5 suspension (approximately 3×10^9 viable organisms) of cells grown on heart infusion agar directly into the crop. The basal diet was an antibiotic-free, practical type ration. Furazolidone was added at the rates of 0.005 and 0.010 percent which is equivalent to 1 and 2 pounds of nf-180* per ton of feed respectively. The medicated feeds were administered continuously after inoculation.

Blood was drawn for serum at the time of inoculation and at 3 or 4 day intervals until the conclusion of the experiment. All experimentally infected chicks were sacrificed at the conclusion of the experiment and *Salmonella* recoveries attempted. Heart, liver, spleen, and gonads were removed, individually macerated in a blendor with tetrathionate broth (Difco) to yield an approximately 10 percent suspension. Blood, bile, rectal swabs and intestinal scraping were put directly into tetrathionate broth. Tetrathionate enrichment cultures were streaked on both brilliant green agar (Difco) and SS agar (Difco). Lactose nonfermenting colonies were picked and transferred to lactose urea agar[6] and lactose non-

* Hess and Clark, Inc., Ashland, Ohio.

fermenting urease-negative cultures were inoculated into carbohydrate broths. Suspected isolates were confirmed by slide agglutination tests.

Serological procedures have been previously described in detail[7]. The tube agglutination test for *S. typhi-murium* was performed with somatic antigen prepared by Dr. J. E. Williams formerly of the Animal Disease and Parasite Research Branch, U.S.D.A. The agglutination tests were read after 24 hours incubation at 37 C. For the indirect hemagglutination test, 0.5 percent chicken erythrocytes (washed three times) were modified (37 C for 30 minutes) by the supernatant fluid (1:20 dilution of 10 X McFarland No. 6 suspension) of heated (100 C for 45 minutes) *S. typhi-murium* cells and washed 3 times in buffered saline. To perform the test, 0.25 ml aliquots of the modified erythrocytes were added to 0.5 ml volumes of serum dilution. Indirect hemagglutination titres were the highest serum dilution in which erythrocytes agglutinated and failed to settle in a compact button after 45 minutes at room temperature. Agglutination and indirect hemagglutination tests were run on aliquots of the same twofold serial serum dilution series. All titres are expressed as the reciprocal of the serum dilution. Significant titres were taken as 1:20 for the agglutination test and 1:160 for the indirect hemagglutination test.[7]

EXPERIMENTAL PROCEDURES AND RESULTS

Experiment 1. A preliminary experiment was performed to observe the effect of furazolidone on weight, mortality, cultural, and serological response of *S. typhi-murium* infected chicks. One hundred day-old cross-bred chicks were obtained and 20 were maintained as the uninfected control. The remaining 80 chicks were orally inoculated with approximately 160 viable *S. typhi-murium* cells per chick, of which 40 were kept as the untreated group and 40 received feed supplemented with 0.01 percent furazolidone. Fifteen chicks from each of the three groups were marked and kept for weight and mortality data. The remaining 25 chicks in each of the two infected groups were used to select five chicks at random for cultures at 3, 6, 12 and 18 days post-inoculation. The results

are summarized in table I. The uninfected and treated groups experienced 7 percent mortality while the untreated groups had 27 percent mortality. Except for an initial weight depression in the untreated group, there were no marked differences in weight gains. *Salmonella* recovery from the intestines of both the untreated and treated groups and from the organs of the untreated group was 100 percent. There were fewer *Salmonella* recoveries from the organs of the treated group, except on the sixth day post-inoculation.

At the conclusion of the experiment, 24 days post-inoculation, eight chicks from the untreated and treated groups were tested with *S. typhi-murium* whole blood plate antigen (supplied by Dr. J. E. Williams), and rectal swab, organ and intestinal cultures were made. The results are given in table II. Although both groups were similarly infected, only the chicks in the untreated group gave a serological response.

Experiment 2. In experiment 1, furazolidone apparently prevented day-old chicks infected with *S. typhi-murium* from becoming reactors to the agglutination test. In an attempt to

TABLE I

The effect of furazolidone on weight, mortality and carrier state of chicks orally inoculated with *Salmonella typhi-murium* at one day of age.

		Days Post-Inoculation				
Criteria	Regime	3	6	9	12	18
Ave. weight gms. (a,b)	Uninfected (e)	53	66	74	100	132
	Untreated (f)	48	57	72	101	127
	Treated (g)	52	68	86	100	132
Mortality percent (b)	Uninfected	0	0	7	7	7
	Untreated	0	7	13	27	27
	Treated	0	0	0	7	7
Organ carriers (c,d)	Uninfected	—	—	—	—	—
	Untreated	5/5	5/5	—	5/5	5/5
	Treated	3/5	5/5	—	4/5	3/5
Intestinal carriers (c)	Uninfected	—	—	—	—	—
	Untreated	5/5	5/5	—	5/5	5/5
	Treated	3/5	5/5	—	5/5	5/5

a, starting weight 41 grams; b, 15 chicks per group; c, 5 chicks at random out of group of 25 for sacrificing; d, liver and heart pool; e, uninoculated untreated controls; f, 160 viable cells per chicks; g, 160 viable cells per chick plus 0.01 percent furazolidone in the feed.

TABLE II

The effect of furazolidone on the carrier and reactor states of *Salmonella typhi-murium* infected chicks.

Regime	Cultures (3)			Whole blood agglutination (3)
	Organs	Intestines	Rectal swab	
Untreated (1)	−	−	−	−
	+	+	−	−
	+	+	−	−
	−	+	−	−
	−	+	+	+
	−	+	+	+
	+	+	−	+
	+	+	+	+
Treated (1,2)	−	+	−	−
	−	+	−	−
	−	+	−	−
	−	+	+	−
	−	+	+	−
	+	+	−	−
	+	+	−	−
	+	+	+	−

(1) 160 viable cells orally per chick at day of age
(2) 0.01 percent furazolidone in the feed continuously
(3) results on 8 chicks per group, 24 days post-inoculation

determine if furazolidone would also prevent a serological response in older chickens, experiment 2 was performed. Thirteen 10-week-old chickens were orally inoculated, six were maintained as untreated controls and seven were maintained on feed supplemented with the "treatment" level of 0.01 percent furazolidone. Serum samples were obtained at 3 day intervals for 30 days. Results of the agglutination and indirect hemagglutination tests are given in figure 1. In the untreated group all chickens developed significant indirect hemagglutinin titres by the 9th day and significant agglutinin titres by the 12th day post-inoculation. Indirect hemagglutinins appeared earlier and were higher in titre than the agglutinins. In the treated group only two chickens developed significant aggutinin titres while all seven exhibited increases in indirect hemagglutinins. At the conclusion of the experiment on the 30th day post-inoculation all birds were sacrificed and cultured. There was only one *Salmonella* recovery from the untreated group which was from the liver of chicken number 4. The two re-

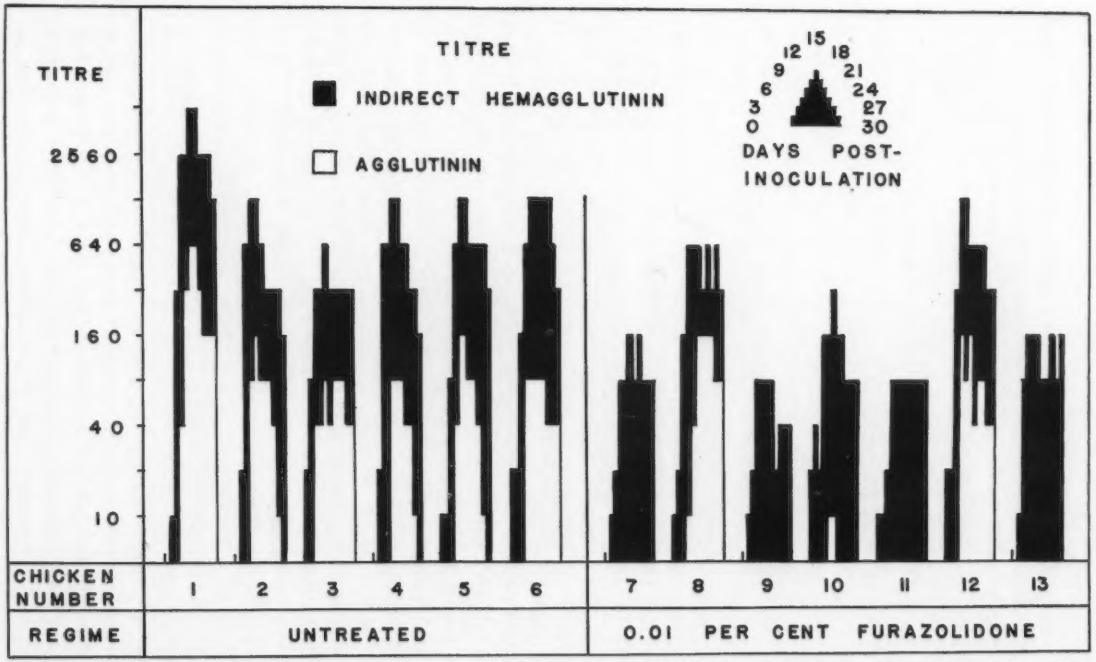

Fig. 1. The effect of furazolidone supplemented feed on the serological response of *Salmonella typhi-murium* inoculated chickens.

coveries from the treated group were from the gonads of number 7 which failed to exhibit an agglutinin titre and from the intestine of number 8 which reacted to both tests.

Experiment 3. In experiment 2, furazolidone treatment at a level of 0.01 percent reduced the number of reactors to the agglutination test but not the indirect hemagglutination test. To confirm this observation experiment 3 was performed. Eighteen 10-week-old cockerels were orally inoculated with *S. typhi-murium;* six were kept as the untreated controls, six were maintained on feed supplemented with a "preventive" level of 0.005 percent furazolidone, and six were fed the 0.01 per cent "therapeutic" level of furazolidone. Serum samples were obtained at 4 day intervals until the 24th day post-inoculation. Results are given in figure 2. Five of the untreated controls developed significant agglutinin titres while one (number 6) had a suspicious titre on day 12 only. All six untreated controls had significant indirect hemagglutinin titres. None of the birds receiving the 0.005 per cent furazolidone level had a significant agglutinin titre although one (number 8) had a suspicious titre on day 12 only. All birds in this group had significant indirect hemagglutinin titres. None of the birds receiving the 0.01 percent furazolidone level exhibited either suspicious or significant agglutinin titres. Although all birds in this group exhibited increases in indirect hemagglutinins, only three could be considered significant while three would be considered suspicious. At the conclusion of the experiment on the 24th day post-inoculation all the birds were sacrificed and only the liver and intestine were cultured. No *Salmonella* recoveries were made from birds receiving furazolidone supplemented feed. The untreated control group had two negative birds (number 1 and 4), three intestinal carriers (numbers 3, 5 and 6), and one liver and intestinal carrier (number 2).

In the preliminary experiment (number 1) furazolidone apparently prevented the reactor state but not the carrier state in chicks infected at day of age with *S. typhi-murium.* Furazolidone treatment of older chickens similarly prevented agglutination reactors but not indirect hemagglutination re-

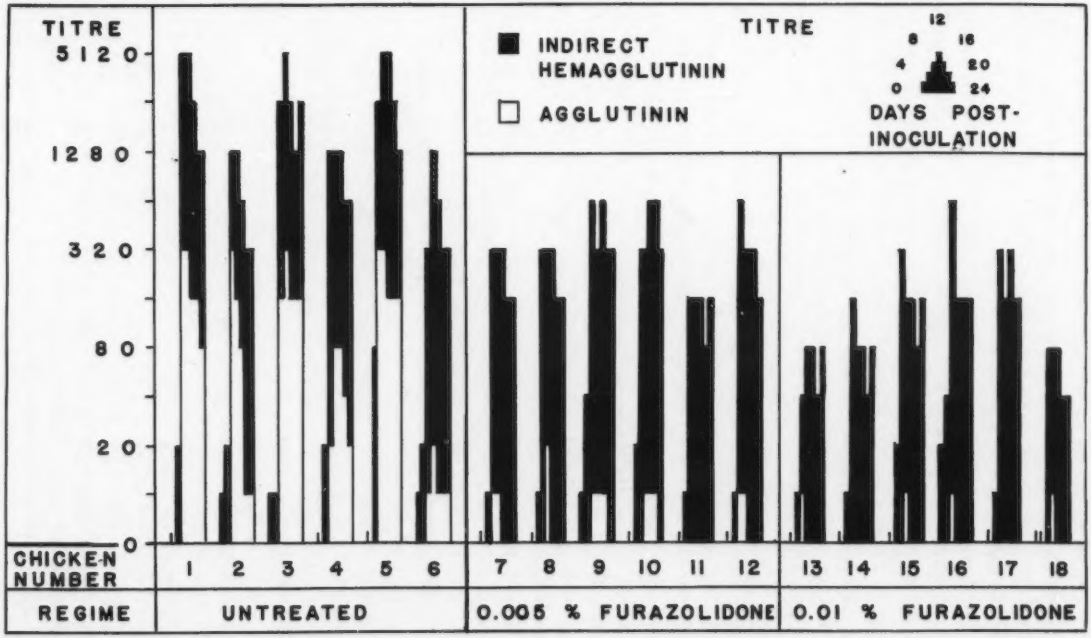

Fig. 2. The effect of furazolidone supplemented feed on the serological response of *Salmonella typhi-murium* inoculated chickens.

actors. Cultures made at 24 and 30 days post-inoculation in experiments 2 and 3 did not reveal any definite information on the effect of furazolidone on the carrier state of older *S. typhi-murium* infected chickens.

Experiment 4. In order to obtain more information on the effect of furazolidone on the carrier state, a fourth experiment was performed. Four lots of 12 ten-month-old laying hens were used. In each lot all 12 hens were orally inoculated; four were kept as untreated controls, four received 0.005 per cent furazolidone supplemented feed, and four received the 0.01 per cent furazolidone level. One lot of four hens from each of the three experimental regimes was sacrificed at 2, 4, 8, and 16 days post-inoculation. Only cultures were obtained from the lots sacrificed at 2 and 4 days. Serum samples as well as cultures were obtained from the lots sacrificed at 8 and 16 days post-inoculation. The results of *S. typhi-murium* recoveries are summarized in figure 3. The number of recoveries from a possible four are given for each recovery site, for each of the three regimes, at 2, 4, 8 and 16 days post-inoculation.

In the untreated regime, recoveries were made from all sites at 2 days only. The most inconsistent recovery was from venous wing blood. Bile recoveries were made at day 2 only. Liver, spleen, ovary, intestine, and rectal swab cultures were obtained from 2 or more birds at 2, 4 and 8 days post-inoculation. At the 16th day post-inoculation only one recovery each was obtained from the spleen, intestine, and rectal swab.

The 0.005 percent furazolidone level reduced the number of recoveries. The blood and bile recoveries were similar to those from the untreated birds. No recoveries were made from the heart. Liver recoveries were made at day 2 only. Spleen and intestine were the only sites from which recoveries were made at 2, 4, and 8 days. No recoveries were made at 16 days post-inoculation.

The 0.01 percent furazolidone level markedly reduced *S. typhi-murium* recoveries, which were obtained from the intestinal tract only. Rectal swab cultures were obtained at day 2 only. Intestinal scrapings yielded cultures from three

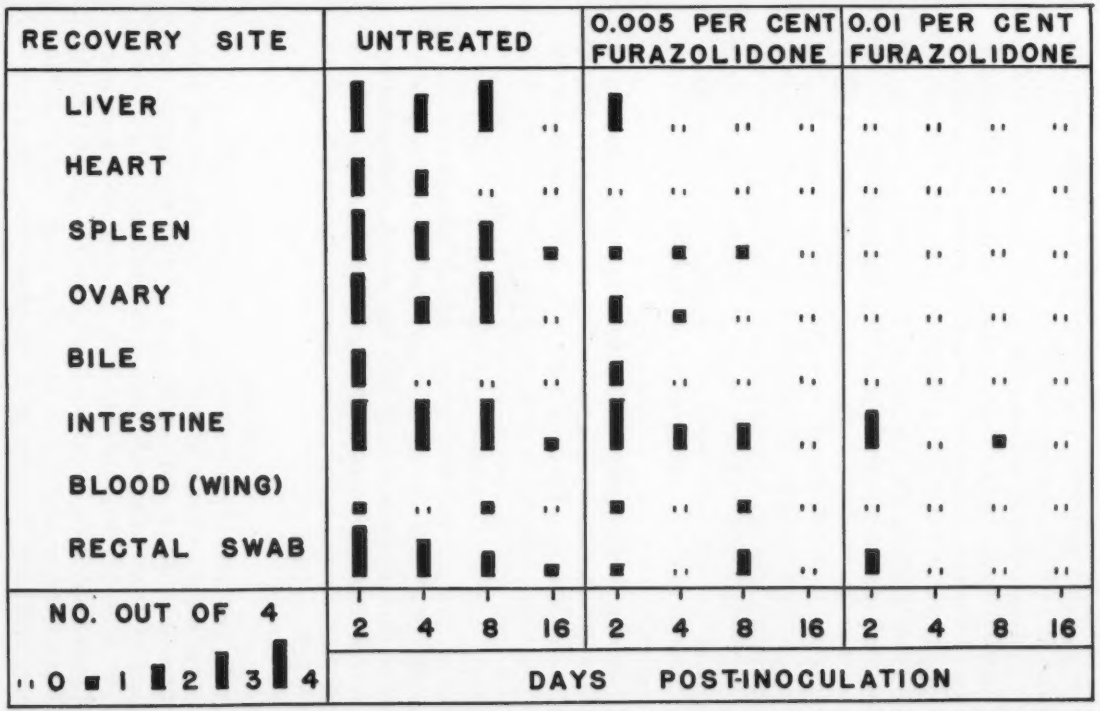

Fig. 3. The effect of furazolidone on the recovery of *Salmonella typhi-murium* from different tissue sites of orally inoculated laying hens.

birds at day 2 and from 1 at day 8. No recoveries were obtained at 16 days post-inoculation.

In an attempt to compare the carrier and reactor states of untreated and furazolidone treated chickens infected with *S. typhi-murium*, the individual results for each bird in experiments 2, 3 and 4 are summarized in table III. The carrier and reactor states did not appear to correlate, even in the untreated groups. At 8 days, four were carriers but only three were reactors. At 16 days there was one carrier which reacted to both serological tests, one which reacted to just the indirect hemagglutination test, and one reactor to both tests which was not a carrier. At 24 days three carriers reacted to only the indirect hemagglutination test while 2 reactors to both tests were not carriers. At 30 days post-inoculation only one out of eight reactors to both tests was a carrier.

In the 0.005 percent furazolidone treated groups, the number of carriers and agglutination reactors were greatly reduced. Carriers were only present at 8 days post-inoculation and both reacted to both serological tests. Seven birds which were not carriers reacted to the indirect hemagglutination test. The chickens receiving the 0.01 per cent furazolidone level also had few carriers and agglutination reactors. Of the three carriers, one was not a reactor, one reacted to both tests, and one reacted to only the indirect hemagglutination test. One reactor to both tests was not a carrier. There were nine reactors to the indirect hemagglutination test which were not carriers.

DISCUSSION

An inoculum of approximately 160 viable *S. typhi-murium* cells per day-old chick was sufficient to cause 27 percent mortality and a 100 percent carrier state in both the organs and intestinal tract. Although feed medicated with the "treatment" level of furazolidone (0.01 percent) reduced mortality to that of the uninfected controls, 100 percent of the intestines and a majority of the organ pools examined contained detectable numbers of *S. typhi-murium*. These obser-

TABLE III

The effect of furazolidone on the carrier and reactor states of individual chickens orally inoculated with *Salmonella typhi-murium*.

Days post-inoculation	Untreated		0.005 percent furazolidone		0.01 percent furazolidone	
	Carriers	Reactors	Carriers	Reactors	Carriers	Reactors
8	+	AH	+	AH	—	—
(expt. 4	+	AH	+	AH	—	—
age 10 mo.)	+	AH	—	—	—	H
	+	—	—	—	+	—
16	+	H	—	—	—	H
(expt. 4	—	—	—	H	—	—
age 10 mo.)	+	AH	—	—	—	—
	—	AH	—	—	—	H
	—	AH	—	H	—	—
24	+	AH	—	H	—	H
	+	AH	—	H	—	H
(expt. 2	—	AH	—	H	—	H
age 10 weeks)	+	AH	—	H	—	H
	+	H	—	H	—	—
	—	AH			+	H
30	—	AH			+	AH
	—	AH			—	—
(expt. 1	+	AH	Not done		—	H
age 10 weeks)	—	AH			—	AH
	—	AH			—	H
					—	—

+ = *Salmonella* recovery
A = Significant agglutination titre
H = Significant indirect hemagglutination titre
— = Negative recovery or titre

vations are in agreement with those of Smith[10] who used an inoculum of 10,000,000 *S. typhi-murium* cells per chick and a furazolidone level of 0.04 percent. Although both the untreated and treated groups in this study were similarly infected at 24 days post-inoculation, only chicks in the untreated group reacted to the agglutination test.

Feed supplemented with both the 0.005 and 0.01 percent levels of furazolidone, which was administered continuously from the time of inoculation, suppressed reaction to the agglutination test but not the indirect hemagglutination test. Smith[11] similarly observed that early furazolidone treatment of fowl typhoid infected chickens prevented the formation of reactors to the agglutination test.

The suppression of agglutinin formation by early furazolidone treatment appears to be due to an elimination of the infection or a reduction in the degree of infection. Concern has been expressed from certain quarters that furazolidone treatment might interfere with pullorum and paratyphoid testing programs. The major fear is that furazolidone treatment of reactors prior to a test might "mask" the agglutination reaction. Gordon and Tucker[3] observed that 0.02 percent furazolidone for 10 days lowered the titre in two out of four birds while the 0.04 percent level rendered seven out of nine pullorum reactors negative. However, Wilson[13] treated 26 *S. pullorum* and *S. gallinarum* carriers with 0.04 percent furazolidone for 12 days and observed no significant or permanent lowering of agglutination titre.

Although *Salmonella* recoveries were obtained from only a few chickens receiving the 0.01 percent furazolidone level, and these were mainly restricted to the intestinal tract, there was a definite increase in indirect hemagglutinins in the majority of the birds. The indirect hemagglutination test for *Salmonella* species has been shown by Neter *et al.*[5] and Sieburth[7] to be more sensitive than the agglutination test. Indirect hemagglutinins appear earlier, attain a higher titre, and persist longer than agglutinins. The increase in indirect hemagglutinins observed in the 0.01 percent furazolidone treated chickens indicates that either the inoculum was suf-

ficient to elicit a response, or that most of the birds had a transient infection which was of insufficient magnitude to be detected by culture or the agglutination test.

Under the conditions of the experiments in this study, where an attempt was made to minimize reinfection, *S. typhi-murium* appears to cause a self-limiting infection of chickens. Almost every tissue site cultured from every untreated bird was infected 2 days after oral inoculation. The number of birds and the number of sites from which *Salmonella* recoveries were made rapidly decreased until only one recovery was made from six birds 30 days post-inoculation. The addition of 0.005 percent furazolidone to the feed apparently hastened the elimination of *S. typhi-murium*. The higher 0.01 percent level mainly restricted the infection to the intestinal tract of those chickens that remained infected.

There was no absolute correlation between the carrier and reactor states in untreated chickens infected with *S. typhi-murium*. Some carriers never became reactors, especially to the agglutination test, while many reactors apparently ceased to be carriers. Under these conditions serological tests appear to be limited to the detection of infected flocks rather than infected individuals.

ACKNOWLEDGMENT

The competent technical assistance of Mrs. Nancy Oakey is gratefully acknowledged.

SUMMARY

1. Chickens maintained on wire in isolation and orally inoculated with *Salmonella typhi-murium* rapidly became infected in many tissue sites, most of which became culturally negative 30 days post-inoculation.
2. There was no correlation between the carrier and reactor states of *S. typhi-murium* inoculated chickens at 8, 16, 24 and 30 days post-inoculation.
3. Furazolidone at a feed level of 0.005 percent appeared to hasten the elimination of *S. typhi-murium* while a level of 0.01 percent apparently restricted the infection to the intestinal tract.

4. Both 0.005 and 0.01 percent furazolidone supplemented feeds suppressed the formation of agglutinins but not the formation of indirect hemagglutinins to *S. typhi-murium* in orally inoculated chickens.
5. A 0.01 percent feed level of furazolidone reduced mortality and prevented the formation of agglutination reactors in day-old chicks orally inoculated with *S. typhi-murium*, but failed to decrease the incidence of intestinal carriers.

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RESPONSE OF EIGHT-WEEK-OLD SUSCEPTIBLE CHICKENS TO NEWCASTLE DISEASE (B-1) AND INFECTIOUS BRONCHITIS VIRUSES

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SUCCESSFUL immunization of a flock of chickens against Newcastle disease (ND) and infectious bronchitis (IB) is dependent upon many factors. Hitchner and Johnson[7] in their original work on the B-1 strain of ND virus reported that chicks vaccinated intranasally at one day of age remained immune to a challenge dose of virus for at least four months. Experiments conducted in this laboratory 5 to 6 years ago indicated that a single dose of vaccine (NDV strain B-1) given to chicks during the first few days of life was capable of inducing an immunity to intramuscular challenge which lasted 10 to 12 weeks.[1] Recent field trials with commercially available ND vaccines employing the same strain of virus administered by drinking water or intranasal routes indicated that chicks vaccinated during the first week of life were found to be susceptible to intramuscular challenge with Newcastle disease virus (NDV) at five to six weeks of age. The change in duration of immunity is believed to be due to the wide use of ND vaccines in breeder flocks, producing offspring with a high and variable passive immunity which

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influences the duration of immunity in the chicks following day-old vaccination.[5,8,9] Another well known factor that influences the production of immunity is the age of the birds at the time of exposure to an antigen.[10] To determine the immunogenicity of commercially available vaccines prepared from the B-1 strain of NDV, and a IB virus an experiment under farm conditions was designed employing susceptible birds 58 days of age when interference of active and passive immunities were nonexistent.

MATERIALS AND METHODS

Experimental birds. The birds in these experiments were White Leghorns obtained as day-old, sexed pullets from a commercial hatchery situated in an area heavily populated with chickens, where ND and IB are endemic and vaccination for these diseases is routinely practiced. Upon arrival, the chicks were placed in an isolation building and were carefully observed by an attendant specifically designated for this operation, until the chicks were 8 weeks of age. On the day the experiment was begun, the birds were transferred to a poultry ranch, typical of California operations. The facilities consisted of a long brooder house divided into five pens by board partitions extending about two feet above the floor and continuing to the rafters with chicken wire. The litter consisted of 2 to 3 inches of wood shavings. The part-time attendant was not aware of the study and took no precautions in feeding and watering the birds. Four other similar buildings for housing layers were situated on the premises within an area of approximately 2 acres. On the 40th post-vaccination day (PVD) the birds were moved from the brooder house into one of the clean laying houses and the daily egg production of each group recorded until termination of the experiment.

Prior to ND vaccination, all birds were tested and found to be negative to the HI test and samples of the flock were found to be completely susceptible to intramuscular challenge doses of ND and IB viruses.

Vaccines. The Newcastle disease and infectious bronchitis vaccines employed in this experiment were available com-

mercially and obtained directly from the manufacturer who recommended the ND vaccine be administered in the drinking water and the IB vaccine intranasally.

Virus. Two strains of ND virus, C and GB, were employed for challenge purposes. The strains were propagated in chicken embryonating eggs and the allantoic and amniotic fluids pooled, distributed in 2 ml ampules and stored at—6 C. The pool was titrated in embryos at 4-week intervals to insure stability of titer. The challenge dose given by the intramuscular and air borne routes in each instance was 200,000 embryo minimal infective doses (mid).

Serology. Serological procedures for ND consisted of the HI test performed as described by Beach.[4] Neutralization tests for IB consisted of serial ten-fold dilutions of IBV in tryptose broth mixed in equal quantities with suspected sera. All sera were diluted 1:10 in sterile physiological saline and inactivated for 30 minutes at 56 C in a water bath. The tubes containing the virus dilutions were kept in iced water bath prior to inoculation of the 9 to 11-day-old embryos. The strain of IBV employed in the test was obtained from Dr. F. S. Markham of the Lederle Laboratories. The virus, upon 3 serial passages through chicken embryonating eggs, consistently caused death within 72 hours.

RESULTS

Newcastle disease vaccination. When the birds were 58 days of age, they were divided into three groups. Groups I and II each contained 101 and 102 pullets respectively which were placed in pens on either side of the control group (III) containing 61 birds. The vaccine was administered to groups I and II in clean, glass, one-gallon, watering jars after all drinking water was withheld from the flock for one hour. Birds in group II drank more avidly than the birds in group I since the weather was warm and the birds in this pen were noticeably more excited. The controls were removed from their pen just prior to vaccination and returned to the house 4 hours later after all of the residual water vaccine had been removed from the premises.

No detectable respiratory signs were observed during the daylight hours of the first 8 days of observation. However, on the ninth day one pullet from group II developed a torticollis. Inoculation of six 9-day-old chicken embryonating eggs with a pooled lung, spleen and brain suspension of the symptomatic chicken did not produce death in the embryos but distinct dwarfing and curling were noted by the 144th hour. The second serial passage of the allantoic and amniotic fluids into 11-day-old embryos resulted in death of four of the six embryos by the 168th hour. Dwarfing and curling of all embryos were observed on examination and the HA titers of the fluids were all 1/2560 or more. No further studies with the isolate were conducted.

As shown in table 1 and figure 1, HI responses were noted in both vaccinated groups by the 10th day, but the HI titers increased significantly by the 22nd PVD. At this time six birds from each group were found to be immune to an intramuscular (IM) challenge of strain C ND virus. No HI antibodies were detected in the non-vaccinated controls (group III) and six birds picked at random were found to be susceptible to IM challenge.

On the 40th day following vaccination, all birds were moved to clean units of one of the laying houses and the control group (III) was divided equally between groups I and II thereby constituting direct contacts. All of the controls were leg banded and designated as groups I-2 and II-2 respectively. In the meantime, another group of 60 three-week-old susceptible chicks were placed in a pen between the two vaccinated groups. This group was designated as group IV.

HI tests and IM challenge of representative birds picked at random from each group at irregular intervals during the succeeding four months, as shown in table I (51, 74, 91 and 120 days following vaccination), indicated that the HI titers of the vaccinated birds dropped considerably. However, serum HI antibodies in low titer were detected in a few birds. Samples of two to six birds removed from each of the vaccinated groups (I and II) on the 51st, 91st and 120th PVD which comprised a sample of 19 birds showed that all but one (on

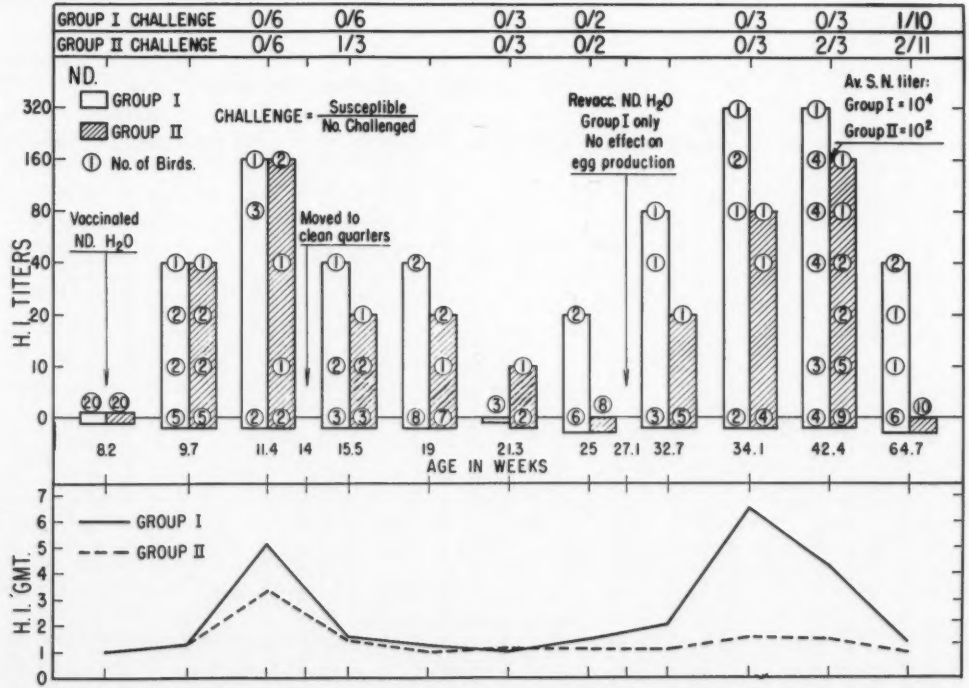

Fig. 1. Serological response and immunity to intramuscular challenge of two groups of susceptible chickens vaccinated with strain B, Newcastle disease virus at 58 days of age.

TABLE I

Serological and challenge results of samples of vaccinated and non-vaccinated chickens removed at intervals during the experiment.

AGE, (Weeks)①		8.2	9.7	11.4		14	15.5		19	21.3		25		27.1	32.7	34.1		42.4		64.7	
PVD⑥			10	22		40	51		74	91		120		132	171	180		239		395	
Number of Birds	Group Number	Treatment	GMT	GMT	②③⑤ Chall.		GMT	③ Chall.	GMT	GMT	③ Chall.	GMT	④ Chall.		GMT	GMT	④ Chall.	GMT	④ Chall.	GMT	④ Chall.
101	I	ND Water	1.3	4.6	0/6	Moved birds to clean quarters. Split group III into I-2 and II-2. Added group IV.	1.6	0/6	1.3	1.0	0/3	1.23	0/2	Revaccinated group I, II, III with B_1 vaccine in drinking water.	2.0	6.5	0/3	4.3	0/3	1.4	1/10
	I-2	None					1.0	1/2	1.0	1.0	2/3	1.0	2/2		2.0	4.9	0/2				
60	III	None	1.0	1.0	6/6																
102	II	ND Water	1.3	3.3	0/6		1.5	1/3	1.15	1.1	0/3	1.0	0/2		1.15	1.6	0/3	1.5	2/3	1.0	2/11
	II-2	None					1.0	1/2	1.0	1.0	2/3	1.0	1/1		1.0	1.0	2/2				
60	IV	None						—	1.0	1.0	—	1.0	6/6		1.0	1.0	3/3	1.0	10/10	1.0	10/10

① Calculated in weeks from age of birds in days. ② All challenged IM with 200,000 mld (emb) using strain C or Boney strain. Approximately an equal number of susceptible controls in each case caused 100% mortality. ③ Strain CNDV. ④ Strain GBND virus.

⑤ Susceptible / No. challenged ⑥ PVD = Post vaccination day, following 1st ND vaccination.

the 51st PVD) were immune to an IM challenge of virulent NDV.

The control group (III) which was placed in direct contact with the vaccinated groups 40 days following the initial vaccination and held as penmates (groups I-2 and II-2) for a period of over 11 weeks showed that 4 of 18 birds were refractory to an intramuscular challenge with a virulent strain of NDV. HI titers were not detected in the sera of these birds.

Nineteen weeks after the initial vaccination when the birds reached 27 weeks of age and were in egg production, group I was revaccinated with drinking water Newcastle disease vaccine. HI titers of the revaccinated birds (group I) increased significantly to a geometric mean titer (GMT) of 6.5 and in the penmates which were not previously vaccinated to a GMT of 4.9 when tested 49 days later. The satisfactory immunological response was verified by a serum neutralization titer 107 days following the 2nd exposure of group I. Twenty sera divided equally into four pools neutralized 10^3, 10^4, 10^4 and 10^5 infectious doses of NDV respectively, whereas four sera of the susceptible penmates vaccinated once presented a titer of 10^2. Revaccination with the ND vaccine showed no clinical evidence of respiratory signs and had no effect on egg production. Serological tests and challenge of birds in the adjoining pens, until the termination of the experiment, indicated that spread of the virus following revaccination did not take place (table 1).

HI tests and challenge studies were continued until the birds were approximately 65 weeks of age, and at this time, no detectable HI antibodies were found in the group of ten birds vaccinated once; however, low titers were still detected in 40 per cent of the birds which were vaccinated twice (GMT of 1.4). Eighty percent of the birds vaccinated once and 90 percent of the birds vaccinated twice were immune to an intramuscular challenge with 200,000 embryo mld doses of the GB strain of NDV.

Route of challenge. On the 21st day following the initial vaccination to ND, six birds of each of groups I and II were

bled and removed to isolation quarters for challenge. Three birds of each group were challenged intramuscularly (IM) and three by the air borne route. The latter method consisted of placing the bird's beak into the opening of a no. 40 DeVilbiss atomizer and squeezing the tube five times. The virus suspension used in each method consisted of a dilution of strain C Newcastle disease virus equivalent to 200,000 embryo lethal doses (eld) per 0.1 ml of suspension.

On the 4th day following challenge, a tracheal swab was made of each bird, and the swab suspended in tryptose broth. Recovery of the virus from the broth (table 2) indicated that

TABLE II

Recovery of ND virus from tracheae of birds vaccinated with ND water vaccine 21 days previously and 4 days following challenge by the intramuscular (IM) and air-borne (AB) routes of inoculation with strain C Newcastle disease virus.

Group	Method of Challenge	HI Titers when Challenged	Results of Challenge[1]	Egg passage of virus recovery
I (Vaccinated)	IM	1/160	0/3	3rd
		1/80		2nd
		1/80		3rd
	AB	1/80	0/3	3rd
		0		2nd
		0		3rd
II (Vaccinated)	IM	1/160	0/3	1st
		1/40		2nd
		1/10		2nd
	AB	1/160	0/2†	3rd
		0		2nd
		0		
III (Non-Vaccinated controls)	IM	0	3/3	1st*
		0		1st*
		0		1st
	AB	0	3/3	1st
		0		1st
		0		1st
IV Susceptible Controls	IM	0	3/3	1st
		0		1st
		0		1st
	AB	0	3/3	1st
		0		1st
		0		1st

[1] Susceptible/Number challenged. †One died of cecal coccidiosis 1 day after challenge. *Two birds died of ND on 4th day; all died by 6th day.

the virus was readily isolated on the 1st embryo passage with tracheal exudate from the non-vaccinated group (III) and the virus control susceptible chicks. Only two of the eleven vaccinated birds yielded the virus on the first embryo passage, but two or three passages through embryonating eggs were required for isolation of the virus from the vaccinated birds. It is evident that the virus was present in the respiratory tract on the 4th day following challenge, irrespective of the method of administration.

Infectious bronchitis vaccination. When the birds were 16 weeks of age (8 weeks following initial vaccination with ND vaccine) groups I and II were vaccinated intranasally with a commercially available IB vaccine. Mild symptoms of IB occurred in group I on the 7th post-vaccination day, but symptoms were delayed in group II and in the unvaccinated control group (IV), to which the virus spread, until the 9th post-vaccination day.

Prior to vaccination, 10 birds picked at random were found to be susceptible to an intratracheal challenge of strain Massachusetts Lg43 infectious bronchitis virus and no SN antibodies were found in their sera. Titers of at least 10^2 or greater were found by the 37th PVD which increased to 10^4 or greater by the 80th PVD in pooled serum samples.

On the 79th day following IB vaccination, when the birds were 27 weeks of age, group I was revaccinated with IB vaccine by the intranasal route. Revaccination had no effect upon daily egg production and no respiratory symptoms were observed in the vaccinated (I, II) or control groups (IV). The immunity determined at intervals by intratracheal challenge with the Massachusetts strain of IB virus showed a solid immunity to IB until the birds were 64.7 weeks of age, the last time the birds were tested.

DISCUSSION

The results of this experiment which simulated field conditions adequately confirm that, in the absence of a parental or residual immunity, a single administration of the B-1 strain of ND virus into birds 8.2 weeks of age was capable

TABLE III

SN titers and results of challenge of birds vaccinated intranasally with a commercial IB vaccine at 16 weeks of age.

Age of birds in weeks	16	21	27		34	44	64.7
	0	37 PVD[1]	79 PVD		128 PVD	194 PVD	337PVD
Group	Chal.[2] Prior Vac.	SN Titer[3]	SN Titer[3]	Chal.[2]	Chal.[2]	Chal.[2]	Chal.
I		10^2 or >	10^4 or >	0/2	0/3	0/3	0/9
I-2		10^3 or >	10^2 or >				
II	10/10	10^2 or >	10^4 or >	0/2	0/3	0/3	0/9
II-2		10^3 or >	10^2 or >				
IV			10^4 or >	0/6			0/9

Revaccinated with IB intranasally

[1] PVD = Post-vaccination day. [2] Challenged with 0.2 ml. undiluted aafl strain Massachusetts Lg43. Susceptible/number challenged. [3] Average of 2 pools of 4 sera each.

of inducing immunity to an intramuscular challenge of virulent NDV in 80 percent of the birds approximately 56.5 weeks later. Such a long lasting immunity with this strain of NDV cannot be expected if given to chicks during the first few weeks of life, which emphasizes the importance of age at the time of initial vaccination and duration of immunity induced by the B-1 strain of NDV.

The immunity induced by strain B-1 given to susceptible birds 58 days of age was sufficient, 19 weeks later, to withstand an intramuscular challenge of virulent virus. Furthermore, revaccination with the B-1 strain did not produce respiratory or nervous symptoms or a drop in egg production.

Similarly, a satisfactory immunity was obtained with IB vaccine when given to the three groups of 16-week-old susceptible birds. Under the conditions of this experiment it was not possible to accurately determine the duration of immunity in the birds following a single exposure to the virus; however, revaccination of one group at 27 weeks of age had no effect upon the respiratory or egg-producing systems of any of the groups of birds which were housed in the same building and all birds picked at random at 64.7 weeks of age were immune to the Massachusetts strain of IB given intratracheally.

Doll, *et al*,[6] concluded from their studies with the B-1 strain of NDV that chicks, when vaccinated at 3 weeks of age and challenged 8 weeks later intranasally, developed an active re-infection of the cells in the respiratory tract. Their data also indicated that recovery of the virus from the trachea was dependent upon the dosage or exposure to the challenge virus. Our observations, as shown by the recovery rate of the virus from the trachea of susceptible and immune birds, following challenge, indicates that a local immunity of the tracheal mucosa occurs in systemically immune individuals but that this immunity is quantitative. The lack of a direct correlation between isolation of the virus and immunity as expressed by HI and neutralizing antibody concentrations in the blood also suggests that the local immunity may vary considerably among individuals or is influenced by some other undetermined factors.[2]

Revaccination under the conditions of this experiment and in the absence of clinical symptoms resulted in a true amnestic response. This was shown by the marked rise in HI titers, increase in neutralization titer and a systemic immunity (90 percent) which persisted until the birds were 64.7 weeks of age. The HI titers (GMT 6.5) in the revaccinated group was not only greater than that following the first vaccination (GMT 4.6) 19 weeks earlier but persisted for a longer period of time (figure 1). It was also interesting to observe that the GMT in the previously non-vaccinated penmates susceptible at 34 weeks of age but vaccinated for the first time was approximately equivalent to the response of the vaccinated birds (GMT 4.9) vaccinated at 8.2 weeks of age.

The chickens used in the experiment comprised the source of hatching eggs for the laboratory which prohibited large samples of birds for bleeding and challenge purposes. Table I was prepared to demonstrate the HI titers of the samples expressed as the GMT of birds picked at random. It is evident that if a line was drawn through the GMT of each group of birds tested, the results would give the reader an erroneous interpretation unless the HI titers of individual birds were also included. Group I, which was revaccinated at 27 weeks, showed a GMT of 6.5 seven weeks following vaccination. One would suspect an adventitious infection as the cause for the amnestic response. This was unlikely because of the irregularity of the HI response among individuals of a small number of birds. The extreme variation and distribution of HI titers among birds of groups I and II were similar and this irregularity and distribution must be considered. The lack of evidence of a field exposure to NDV is also substantiated by the susceptibility of the unvaccinated controls. It is evident therefore that the amnestic response as shown in table 1 and figure 1 is a valid interpretation and that the GMT expressing the HI response can be misleading sometimes, particularly following the administration of strains of NDV which do not produce a high HI response with regularity. It is axiomatic that this can be obviated by the use of large numbers in the sample.

The low potential of spread of a virus in a vaccination program is a definite advantage. There was no clinical or serological evidence in the present trial that spread of the virus to the house controls (group IV) occurred; however, 4 of 13 susceptible birds placed as penmates with the principals 40 days after vaccination and kept in contact for 11 weeks were immune to IM challenge. This may indicate that a low degree of spread occurred. The absence of symptoms and serological (HI and SN) response of chickens immune to challenge to exposure to NDV have been previously observed.[2,9] It is doubtful that the vaccinated birds, based upon the low potential of spread of the B-1 strain of virus in this experiment, were continually exposed to this virus in the environment. The results of this experiment, therefore, emphasize that a longer immunity can be expected when susceptible chicks are vaccinated at an age when the birds are capable of responding. This is applicable even when a virus of relatively low virulence is applied by one of the mass techniques.

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SUMMARY

Vaccinating 8-week-old replacement chickens for egg production under controlled field conditions demonstrated the influence of age and passive immunity upon immunogenicity of a commercially available B-1 strain of Newcastle disease and infectious bronchitis vaccine.

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STUDIES ON EXPERIMENTAL EGG TRANSMISSION OF PLEUROPNEUMONIA-LIKE ORGANISMS IN CHICKENS*

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CHRONIC respiratory disease (CRD) of chickens has become, in recent years, a condition of great economic importance to poultrymen and, therefore, has commanded the time and interest of many poultry pathologists. It is generally conceded that pleuropneumonia-like organisms (PPLO) are intimately associated with CRD if not the basic cause.

Egg transmission of chronic respiratory disease (CRD) has been incriminated by several workers as one of the more important means of dissemination of the CRD agent and there has been considerable evidence presented which points toward its occurrence. Much of it is circumstantial. Van Herick and Eaton[10] were first to discover PPLO in fertile hens' eggs accidently while working with the primary, atypical, pneumonia virus of humans in chick embryos. Jerstad and Hamilton[5] found turkey eggs, produced by a pen of infected breeders, contaminated with the agent of infectious sinusitis. These workers followed up their findings by inoculating fourteen-day turkey embryos (from non-infected dams) with PPLO and hatching the poults. They isolated the agent from a day old poult that died. Five poults developed sinusitis between 6 and 9 weeks of age. Similar results were recorded by Mataney *et al.*[8]

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The examination of cull chicks and pipped embryos from CRD infected flocks by Van Roekel and Olesiuk[12] revealed that ten percent of cull chicks and 46 percent of pipped but unhatched embryos produced by an infected supply flock had lesions of the respiratory tract characteristic of CRD No attempts to isolate PPLO were made. They noted[13] that the presence of the agent in chicks at the time of hatching did not result in a natural outbreak of the disease later on. In a subsequent report,[11] successful CRD transmission was recorded when a stress in the form of infectious bronchitis was applied to the chicks. Cover and Waller[1] also examined pipped and dead embryos from a commercial hatchery and found 72 percent were infected with the CRD agent. Fahey and Crawley[2] followed the breeding stock of one hatchery through four generations, each of which developed symptoms of CRD and yielded PPLO on culture. The authors mentioned that egg transmission appeared to be the major means of spread of PPLO.

This study was undertaken in an effort to produce an experimental means of egg transmission of PPLO that would be reproduceable. If this could be accomplished, further studies concerning the importance of stress, treatment, and numerous other factors, would be greatly facilitated. Experiments were done according to the following general plan: 1. The survival time of PPLO in fertile, infertile, and embryonating eggs subsequent to various incubation and holding conditions was determined. 2. Attempts were made to recover PPLO from the tissues of chicks hatched from eggs that had been inoculated with PPLO as fresh eggs or as five day embryos. 3. Chicks hatched from PPLO inoculated eggs were reared in isolation and stressed with infectious bronchitis virus at a later date in an attempt to produce CRD. 4. Non-infected laying fowls were inoculated via the intraperitoneal, intravenous, or intraoviduct route and their eggs subsequent to the inoculation examined for the presence of PPLO.

MATERIALS AND METHODS

PPLO strains and their characteristics. PPLO strains 85, 39, 293 and CB were used in the experimental work pre-

sented here. The first three were isolated from tracheas of field cases diagnosed as CRD. They were identified as PPLO on the basis of fermentation reactions in liquid media[4] (acid, no gas in dextrose, no acid in lactose), colonial characteristics on solid media as described by Lecce and Sperling[6] and morphological characteristics as described by Freundt.[3] PPLO strain CB was an egg adapted strain (nearly 200 consecutive yolk sac passages in eggs) that killed 100 percent of the embryos inoculated within 2 to 3 days.

Yolk sac inoculations with strains 85 and 39 caused an irregular mortality pattern with abscesses in the tibiotarsal articulations or necrotic areas in the liver in occasional embryos. Deaths occurred between the third and twelfth days postinoculation.

When inoculated into the air sacs and tracheas of adult birds neither 85 (175th liquid media transfer) nor 39 (4th transfer) caused respiratory symptoms or produced gross lesions typical of CRD. Strain 293 (6th transfer), however, did produce both respiratory symptoms and severe lesions in two adult hens when inoculated into the peritoneal cavity. The air sacs were thickened and contained much fluid and solid yellow exudate, the lungs had areas of consolidation, and exudate was present in the tracheas of these birds. One bird died five days postinoculation and the other was destroyed 19 days postinoculation. Strain 85 (42nd transfer) caused neither respiratory symptoms nor gross lesions during 68 days observation following intrasinusoidal inoculation of turkeys.

Liquid media techniques. The liquid media employed in this study was developed by Grumbles, *et al.*,[4] and modified by Taylor.[9] It consisted of a phenol red broth base medium containing one percent dextrose or lactose, one percent PPLO serum fraction, 1,000 units of penicillin per ml and a 1/1,000 concentration of thallium acetate. Henceforth it will be referred to as PRBB.

Tubes containing 3 ml were ordinarily seeded with 0.2 ml of inoculum. The lactose cultures served as inoculum for eggs, birds, and culture media. PPLO strains 85, 39, and 293

were maintained by transfers to fresh media at regular intervals. Occasional cultures were checked by Gram stained smears for the presence of typical PPLO forms and the absence of contaminants. Cultures resulting from yolk or tissue inoculums were transferred serially three or four times before smears were examined to avoid the presence of confusing debris from the original inoculums.

Titrations were done by making tenfold, serial dilutions in sterile nutrient broth and culturing the dilutions in dextrose PRBB. Acid formation and the typical morphology in Gram stained smears were the criteria used to determine the end point.

Methods of inoculation and isolation of PPLO. Fresh eggs were inoculated by the usual manner into the yolk with 0.1 ml of lactose PRBB (without thallium acetate) culture diluted 10^{-1} in sterile nutrient broth. Isolation or recovery of PPLO from fresh eggs was done in several ways. Either the entire contents or only the yolk of the egg were poured into approximately 90 ml of sterile nutrient broth contained in an eight ounce screw cap jar. After thorough shaking, the mixture yielded a dilution of yolk roughly equivalent to 10^{-1} which served as inoculum for subsequent cultures. Embryonating eggs were cultured either in the same manner (the presence of the embryo did not interfere with the mixing of the yolk) or by removing a one to two ml sample of yolk directly with a sterile pipette and diluting it tenfold.

Preparation of inoculums from embryos, chicks, and adult birds. Lung, trachea, and yolk sac were used for PPLO isolation attempts from chicks. One lung together with the lower half of the trachea (removed aseptically) in one to two ml of broth were ground in a TenBroeck tissue grinder. One to two ml of yolk sac and adhering yolk were removed and a portion shaken with one to two ml of broth in a test tube. The resulting mixtures were used for inoculating liquid media. Only the yolk and yolk sac were cultured from dead and pipped embryos.

Sterile cotton swabs were used to collect exudate from the

lumen of the trachea or from the peritoneal and air sac cavities of adult chickens. Adhering exudate and debris were shaken off in a tube containing two ml of broth and aliquots of the mixture were used for culturing.

Experimental chickens and eggs. The experimental chickens used in this work were obtained as 14 and 17-week-old White Leghorn pullets from the experimental farm flock which had been maintained free of all respiratory infections and immunizations. Prior to experimentation, they were placed in laying batteries and successfully immunized against infectious bronchitis, Newcastle disease, and fowl pox. Three White Leghorn roosters kept in the batteries supplied semen for artificial insemination during the course of experimentation.

Fertile eggs were obtained from the supply flock on the experimental farm. Infertile eggs came from non-infected pullets held in laying batteries. Chicks for experiment were hatched from eggs produced by the disease free station flock and were held in modified Horsfall units.[7]

Incubation and holding conditions of eggs. Trials were conducted in which eggs were stored under two different sets of conditions. In one case, they were kept in a room in which the temperature was maintained at 58 to 60 F and the relative humidity at approximately 78 percent. In the other case, an insulated box was used in which temperatures and humidities ranged from 77.0 to 84.2 F (average 81.6 F) and 71.5 to 81.0 percent (average 75 percent) respectively.

Infertile and fertile eggs not intended for hatching were held in a bacteriological incubator at 37 C. Eggs for hatching were incubated in a commercial machine and turned twice daily. Broth inoculated control eggs were always hatched in a separate incubator.

Surgical techniques. Anesthesia when necessary, was effected by intravenous injection of pentobarbital sodium (one grain per ml) or a combination of pentobarbital sodium given intravenously and one percent hexylcaine hydrochloride given locally.

Intraperitoneal inoculations were made by entering

through a surgical incision in the abdominal cavity between the 5th and 6th ribs on the left side and depositing the inoculum directly on and around the ovary. Intraoviduct inoculations were also made through a surgical opening between the left pubis and the point of the bend in the last left rib. The inoculum was injected into the lumen of the oviduct.

Intravenous inoculations were made by injecting the inoculum into the superficial ulnar vein. Semen was contaminated with PPLO either by washing colonies off agar plates with fresh semen or by diluting the semen with equal volumes of PRBB culture.

RESULTS

Viability of PPLO in the yolks of fresh infertile eggs incubated at 37 C. Three trials were done to determine the viability of PPLO strain 85 in the yolks of infertile eggs incubated at 37 C. In trial 1, the 31st lactose PRBB transfer was used to inoculate 20 fresh infertile eggs. Ten eggs were inoculated with sterile nutrient broth to serve as controls. Trial 2 involved 30 eggs inoculated with the 145th lactose transfer and trial 3, 30 eggs inoculated with 312th transfer. Ten broth-inoculated controls were employed in each of these trials.

PPLO strain CB was used to inoculate 12 infertile eggs in trial 4, with 4 eggs serving as broth-inoculated controls. The results of trials 1 to 4 are summarized in table 1.

It should be noted that in the three trials done with PPLO 85, there was no survival after 4 days incubation. In trial 4, an endpoint was not reached. However, titers dropped about one log after 4 days incubation. The fact that this was an egg adapted strain might explain the difference in results from those obtained with strain 85. All broth-inoculated controls were negative for PPLO.

Viability of PPLO in the yolks of fresh, fertile eggs incubated at 37 C. Six trials were done to determine the viability of PPLO in the yolks of fertile eggs incubated at 37 C. Some of the trials were rather short and incomplete due to overheating of the incubator in one case and a rather high, early, embryo mortality in all cases. The high embryo mortality was due to

TABLE I

The effect of duration of incubation at 37 C on the viability of PPLO 85 and PPLO CB inoculated into the yolks of fresh infertile eggs.

Duration of Incubation	Trial 1 Str. 85 Trans. 31	Trial 2 Str. 85 Trans. 145	Trial 3 Str. 85 Trans. 312	Trial 4 Str. CB Yolk
2 hours	1* 10^{-3} 2 10^{-4} 1 10^{-5}	2 10^{-2} 4 10^{-3}	1 10^{-2} 5 10^{-3}	2 10^{-2} 1 10^{-3}
1 day	1 10^{-1} 2 10^{-2} 1 10^{-4}	None Cultured	4 10^{-2} 2 10^{-3}	1 10^{-2} 2 10^{-3}
2 days	2 Neg. 2 10^{-2}	6 Neg.	4 Neg. 1 10^{-1} 1 10^{-2}	1 Neg. 2 10^{-2}
4 days	3 Neg. 1 10^{-2}	6 Neg.	6 Neg.	2 10^{-1} 1 10^{-2}
8 days	4 Neg.	6 Neg.	6 Neg.	None Cultured

* Numbers = the number of eggs yielding the titer indicated.

the mechanical injury inflicted during the inoculation procedure since approximately equal mortality occurred in broth inoculated controls. An experiment designed to check on the possible harmful effect of the penicillin used in the inoculum did not incriminate the antibiotic.

Fresh fertile eggs were inoculated into the yolks with PPLO 85 (39th to 68th lactose transfers), incubated at 37 C and cultured and titrated at various intervals. Broth-inoculated controls were incubated and cultured at the same intervals. Generally, as the duration of incubation increased, the percentage of PPLO recoveries dropped but the PPLO titers in eggs supporting growth increased. Twenty-nine broth-inoculated control eggs were free from PPLO. Results in the 6 trials are found in table 2.

Viability of PPLO in the yolks of eggs stored at 58-60 F and at 80 F. Three trials were done to determine the effect of storage on the viability of PPLO in the yolks of eggs. In trial 12, fresh fertile eggs were inoculated with PPLO 85 (109th transfer) and were stored at 80 F and 75 percent relative humidity. Storage at 58 to 60 F and 78 percent relative

TABLE II

The effect of incubation at 37 C on the viability of PPLO 85 inoculated into the yolks of fresh, fertile eggs.

Duration of incubation at 37C.	Trial 5 Str. 85 39th trans.	Trial 6 Str. 85 51st trans.	Trial 7 Str. 85 57th trans.	Trial 8 Str. 85 64th trans.	Trial 9 Str. 85 66th trans.	Trial 10 Str. 85 68th trans.
1-5 hrs.	1* 10^{-1} 3 10^{-2}	2 10^{-2} 4 10^{-3}		2 10^{-2} 2 10^{-3}	4 10^{-3}	2 10^{-2}
1 day	1 Neg. 1 10^{-1} 1 10^{-2} 1 10^{-3}					
2 days	1 10^{-1} 2 10^{-2} 1 10^{-4}					
4 days	1 Neg. 1 10^{-3} 1 10^{-4} 1 10^{-5}	2 Neg. 1 10^{-1} 2 10^{-3} 1 10^{-5}	3 Neg.	3 Neg. 1 10^{-2}	3 10^{-4} 1 10^{-5}	2 Neg. 2 10^{-2}
7-8 days	2 Neg. 1 10^{-2} 1 10^{-6}			1 Neg. 1 10^{-5}		2 Neg. 2 10^{-2} 1 10^{-6}
12 days			1 10^{-6}			
16 days			2 Neg. 1 10^{-5} 2 10^{-7}			

* Numbers = the number of eggs with the corresponding titer.

humidity was used in trials 13 and 14. Fresh infertile eggs inoculated with PPLO 85 (90th transfer) and fresh fertile eggs inoculated with PPLO 85 (120th transfer) were used in the two trials respectively. A total of 22 broth-inoculated controls were cultured in these trials. No PPLO isolations were made. Data from trials 11 to 13 are presented in table 3. Storage of eggs at 80 F did not kill the PPLO within 7 days, the longest period tested. PPLO remained viable for 8 to 19 days and 24 to 28 days in the trials conducted at the lower temperature.

TABLE III

The effect of duration of storage at 80 F, 75 percent relative humidity and at 58 to 60 F, 78 percent relative humidity on the viability of PPLO 85 inoculated into the yolks of fresh, fertile and infertile eggs.

	80 F, 75% Rel. Hum.	58 F, 78% Rel. Hum.	
Duration of Storage	Trial 11 Fert. eggs Str. 85 109th trans.	Trial 12 Infert. eggs Str. 85 90th trans.	Trial 13 Fert. eggs Str. 85 120th trans.
1 to 4 hrs.	1* 10^{-2} 1 10^{-3}	4 10^{-2} 2 10^{-3}	1 10^{-1} 5 10^{-2}
4 days	3 10^{-1} 3 10^{-2}	1 Neg 2 10^{-1} 3 10^{-2}	3 10^{-1} 3 10^{-2}
7 to 8 days	3 10^{-1} 3 10^{-2}	5 10^{-1} 1 10^{-2}	1 Neg 2 10^{-1} 3 10^{-2}
12 days			1 Neg 2 10^{-1} 3 10^{-2}
16 days			3 Neg 2 10^{-1} 1 10^{-2}
19 to 20 days		6 Neg	5 Neg 1 10^{-1}
24 days			3 Neg 3 10^{-1}
28 days			6 Neg

* Numbers = the number of eggs with the corresponding titer.

Transmission of PPLO to chicks by inoculation of eggs. In trial 14, 46 fresh, fertile eggs and 19 five-day embryos were inoculated with PPLO 85 (95th transfer) into the yolk or yolk sac, incubated and hatched. Chicks were held in modified Horsfall isolation units for two weeks and then cultured for PPLO. Broth-inoculated and uninoculated eggs were hatched as controls. PPLO isolation attempts were made at hatching time from six live chicks, six pipped embryos, and eight dead embryos in shells. At hatching time one isolation from each group was obtained. None of the six chicks cultured at 2 weeks yielded PPLO. In the group inoculated as 5-day-embryos, PPLO were isolated in each of 10 attempts at hatching time. At two weeks of age, one out of six chicks yielded PPLO on culture. This infected chick had the only gross lesions seen in the experiment, a concurrent mold infection and an aerosacculitis. All isolation attempts from 31 broth-inoculated and uninoculated control eggs failed both at hatching time and at 2 weeks of age.

In trial 15, five-day embryos were inoculated into the yolk sac with PPLO 85 (144th transfer) and hatched. Broth-inoculated embryos were hatched as controls. The chicks were held in isolation units. Samples from each group were examined and cultured at hatching time and at two weeks of age. At this time, all broth controls and half of the PPLO-inoculated group were given infectious bronchitis virus. Subsequently, a mild respiratory reaction was seen. There was no mortality. At 3½ and 4 weeks of age, chicks were again examined for lesions and cultured for PPLO. Detailed information is given in table 4.

It should be noted that although no PPLO isolations were made after one day of age, the PPLO-inoculated, bronchitis-stressed group did show some evidence of air sac infection in the form of yellow fluid and solid exudate in the air sacs and slightly to moderately thickened air sacs. The modified Horsfall units used for holding these birds were dry, warm, and excellently ventilated. Such conditions were certainly less favorable for an outbreak of CRD than in most broiler houses.

TABLE IV

Trial 15—The effect of inoculation of PPLO 85 into five-day embryos on the resultant hatch of chicks that were stressed with infectious bronchitis virus at 2 weeks of age.

Group	Day old chicks			2 week old chicks			3½ week old chicks			4 week old chicks		
	PPLO isol.	Sympt.	Les.	PPLO isol.	Sympt.	Les.	PPLO isol.	Sympt.	Les.	PPLO isol.	Sympt.	Les.
1. PPLO into five-day embryos	10/10	None	None	0/10	None		0/5	None	None	0/5	None	None
2. PPLO into five-day embryos— inf. bron. 2 wks. of age							0/5	++5	±3 +2	0/5	±5	±1 +3 ++1
3. Broth into five-day embryos inf. bron. 2 wks. of age	0/5	None	None				0/5	++5	±5	0/5	—4 +1	—1 ±4

Numerators = Number of PPLO isolations. Denominators = Number of chicks cultured.
Numbers indicate the number of chicks examined that had symptoms or lesions indicated.
Symptoms = — Negative, ± an occasional respiratory "click", + frequent "clicks", ++ obvious coughing, sneezing, and nasal exudate.
Lesions = — Negative, ± slight fluid exudate or a few flecks on air sacs, + clouding or thicking of air sacs, ++ liquid or solid exudate in air sacs.

Attempts at egg transmission of PPLO by inoculation of the reproductive tract. Twenty-one adult birds were kept in individual laying cages and were injected with or exposed to PPLO. Strain 85 was used to inoculate two hens intravenously, five hens intraperitoneally, three hens by insemination with PPLO contaminated semen, and three hens by the intraoviduct method (inoculation directly into the lumen of the upper oviduct). Strain 39 was used in two intravenous and two intraperitoneal inoculations, and strain 293 in one intravenous, two intraperitoneal, and one intraoviduct inoculation.

Eggs produced prior to and following exposure were examined for PPLO. Altogether 559 eggs were examined of which 217 were laid before infection. An average of 26.3 eggs per hen were cultured. PPLO were not recovered from any of the eggs. With three exceptions, there were no noticeable effects from the inoculations. The two hens receiving strain 293 intraperitoneally developed severe air sac infections typical of CRD and one died five days after inoculation. One bird inoculated with strain 85 intraperitoneally ceased laying for 2 weeks. No symptoms were noted in this bird, however.

DISCUSSION

In studying the survival of PPLO in the yolks of eggs, several interesting facts were noted. It was found that infertile eggs were incapable of supporting the growth of PPLO strain 85 and viability of the PPLO was of short duration at incubator temperature (less than 4 to 8 days). An egg adapted strain (CB) apparently lived better at incubation temperature though titers were falling off after 7 days, the longest period tested.

In fertile eggs, on the other hand, PPLO remained viable for the full 21-day incubation period and also multiplied. Titers rose markedly from the 5th to 7th day on. During the first 3 to 4 days, the PPLO titers in the inoculated yolks remained constant or declined slightly and in some eggs the inoculum died. It would appear that the first 3 to 4 days incubation is a critical period in the survival of PPLO in the

yolks, at least in eggs experimentally inoculated.

In view of our findings one might suspect that merely holding eggs before incubation would eliminate viable PPLO. It was found however, that PPLO remained viable for 24 days when stored at 58 to 60 F and for at least 7 days storage at 80 F. Storage of eggs for longer than these periods at the temperatures tested has a very detrimental effect on subsequent hatchability and thus renders this means of eliminating viable PPLO useless from a practical standpoint. If holding conditions could be found that would not affect hatching adversely, additional experiments along this line would be worthwhile in view of the fact that after 28 days all PPLO were dead.

Fresh hatching eggs inoculated with PPLO produced chicks 15 percent of which yielded PPLO on culture. Five-day embryos inoculated and then allowed to hatch yielded 100 percent recoveries. This again indicates that a critical period exists in the growth of PPLO during the first 5 days incubation of an embryonating egg. The fresh egg inoculations definitely proved that PPLO can exist within an egg for the full 21-day incubation period, though the 5-day-embryo inoculation procedure was obviously the method of choice in producing PPLO infected chicks in any number.

When infected chicks were raised in isolation, it was found that although culturable PPLO were absent at 2 weeks of age, some indication of infection could be established by stressing the birds with infectious bronchitis virus. Thickened air sacs and some solid exudate were found following the stress, both of which are commonly associated with CRD. The lesions were not severe. It is likely that the selection of a more pathogenic PPLO strain would have produced a more severe infection.

Results with intravenous, intraperitoneal, and intraoviduct inoculations of PPLO into adult birds were not successful in effecting experimental egg transmission of PPLO. The rate of natural transmission appears to be low, so that possibly there were too few attempts made. Other possible reasons for our failure might be that our strains were relatively

non-pathogenic, the experiments were conducted under stress-free conditions, and, that PPLO may infect the egg by means other than those tried here.

SUMMARY

1. The viability of a strain of pleuropneumonia-like organism (PPLO) in the yolks of hen eggs was studied. It was found that when incubated at 37 C, the PPLO died in infertile eggs between the 4th and 8th day. PPLO persisted and grew in fertile eggs. When stored at 58 to 60 F, PPLO inoculated into the yolks of fertile eggs remained viable for 24 days. At 80 F, viability was maintained for at least 7 days.

2. Experimental egg transmission was induced by the inoculation of both fresh eggs and 5-day-embryos with PPLO. One out of six of the chicks that hatched from inoculated fresh eggs and four out of four of the chicks from the embryos inoculated at 5 days were infected with PPLO in the respiratory tract. Chicks from the latter group, when held in isolation did not have symptoms of CRD until after infectious bronchitis virus was administered at 2 weeks of age. Following this stress, lesions of air sac infection were seen in four out of five chicks but PPLO were not recovered from the birds.

3. Twenty-one adult hens were exposed to three different strains of PPLO by the following routes of administration: intraperitoneal, intravenous, and intraoviduct injections. Insemination with infected semen was also done. Two hundred and seventeen eggs produced before exposure and 342 eggs laid after infection by these treated birds were incubated and cultured for PPLO. In no instance were PPLO recovered from any egg.

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CASE REPORT—PPLO INFECTION IN CHUKAR PARTRIDGES (ALECTORIS GRAECA)

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IN December of 1956 a group of near mature chukar partridges were submitted to this laboratory from a game farm located in central Nevada with the complaint that the

birds were "droopy and rubbed their eyes on their wings." There were about 1600 partridges and 200 pheasants on the farm.

The partridges were divided into two flocks; a breeding flock which contained 800 birds in one large isolated pen and a growing flock. The growing flock in which the disease problem was present, originally consisted of 800 birds of which approximately one-third had died. This flock was divided among four of five adjoining pens which were recently constructed on ground that had not been previously used for raising birds. The fifth adjoining pen contained pheasants. Neither the pheasant nor partridge breeding flock showed evidence of disease.

The six live and twelve dead partridges submitted were emaciated. Three of the live specimens and eight of the dead specimens exhibited marked swelling of the infraorbital sinuses (fig. 1 and 2). Sera collected from these birds agglutinated an antigen prepared from the S6 pathogenic strain of PPLO. On necropsy, a mild tracheitis was present in several cases while the lungs, air sacs and viscera appeared normal except in one instance, in which a heavy infection of *Capillaria sp.* was observed in the crop. A hemorrhagic enteritis was also evident in this specimen.

The sinus exudate and tracheal scrapings were cultured on PPLO agar (Difco) supplemented with 10 percent horse serum and 1 percent yeast hydrolysate and incubated at 37 C. PPLO colonies were observed within 24 hours. The colonies stained readily by the method of Dienes.[3] Broth cultures of the PPLO were not lethal for 6-day-old embryonating chicken eggs when inoculated into the yolk sac; however, PPLO was demonstrated by sub-culture of yolk on serum agar.

Pooled sinus exudate from the affected chukar partridges after storage at —76 F for two months was inoculated into the infraorbital sinus and abdominal air sacs of four turkey poults. No symptoms were observed during a 17-day observation period and no lesions were found on autopsy. Attempts to re-isolate PPLO from the sinuses and air sacs of the inocu-

Fig. 1 Extensive swelling of infraorbital sinuses of a chukar partridge with PPLO infection.

Fig. 2 Lateral view of swollen sinus of chukar partridge. Note matting of feathers on wing.

lated poults were negative. Six turkeys were inoculated into the sinuses and air sacs with egg yolk material from embryonating chicken eggs that had been inoculated with broth-cultured PPLO. Very mild air sac lesions were observed at autopsy in two of the turkeys inoculated The sera of two of these turkeys agglutinated antigens prepared from the S6 strain of PPLO pathogenic for turkeys and chickens while none of the sera were positive to the non-pathogenic PPLO antigenic types.

The histopathology of the infraorbital sinuses of the partridges was similar to that described by Jungherr[4] in turkeys. The mucosal epithelium showed marked thickening with hyperemia and edema. The edema extended into the submucosa and a moderate mononuclear infiltration and massive lymphofollicular nodules were observed.

PPLO isolated on enriched agar plates from the partridges were subjected to antigenic typing.*[2] Two antigens[1] prepared from individual colonies picked from enriched agar plates were agglutinated by PPLO hyperimmune rabbit serum prepared from "nonpathogenic" antigenic types, while no agglutination occurred in the presence of specific hyperimmune rabbit serum prepared from pathogenic types of PPLO. Fermentation reactions were tested in phenol red broth (Difco) enriched with 10 percent horse serum and buffered to pH 7.8. Two reactions were observed during an eight day incubation period, one in which dextrose, sucrose and maltose were utilized with the production of acid and no gas while lactose and mannite were not fermented and a second reaction in which no fermentation occurred in the sugars tested.

It is interesting to note that sinus exudates collected from the affected partridges failed to produce any observable disease when inoculated into the sinus and air sac of turkey poults while yolk material, from embryonating chicken eggs† which had been inoculated with PPLO broth subcultures, caused a mild disease when inoculated into turkey poults. An explan-

* The author is grateful to Dr. H. E. Adler for typing the culture.

† Fertile chicken eggs employed in this work were collected from a department flock free of PPLO infection.

ation for this may be that the PPLO present in the sinus exudates did not survive storage or too few organisms may have been present.

It is also noteworthy that antigens prepared from the PPLO isolated on enriched agar were of the nonpathogenic antigenic type of PPLO. Subcultures of these isolates when grown in embryonating chicken eggs and inoculated into the sinus and air sacs produced lesions in the air sacs and a response of antibodies which agglutinated an antigen prepared from a known pathogenic type of PPLO. An explanation for this may be that enriched serum agar may be more selective for the nonpathogenic types of PPLO while embryonating chicken eggs are more selective for certain pathogenic strains of PPLO. It is unfortunate that chukar partridges were not available for passage of the original exudate since species susceptibility to the strains of organisms encountered may have been involved.

As far as it is known, this is the first reported case of PPLO infection observed in chukar partridges; however, a report of a PPLO infection in partridges of unreported species was made by Jungherr in 1952.[5]

SUMMARY

A sinusitis in chukar partridges caused by a pleuropneumonia-like organism has been described.

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CASE REPORT — *TETRAMERES AMERICANA* (CRAM, 1927) INFECTION IN DOMESTIC PIGEONS

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THE purpose of this paper is to describe a natural outbreak of *Tetrameres americana* in domestic pigeons in California. Cram,[3] in 1931, reported an experimental infection of one pigeon with larvae of *T. americana*. Natural *Tetrameres* infections in pigeons were reported in Morocco by Santucci *et al.*,[5] in France by Bechade and Bechade,[2] in Italy by Romboli,[4] and in Portugal by Alves da Cruz and Rodrigues da Costa.[1] In a paper dealing with pleuropneumonialike organisms infection in pigeons which were submitted to this laboratory in June 1954, Mathey *et al.*[6] mentioned the presence of this parasite. As far as the writers were able to determine, this is the only previous report of a natural *T. americana* infection in domestic pigeons in this country.

On 5 Dec 1953, four dead pigeons of a flock of 65, approximately 18 months of age, were submitted to this laboratory for diagnosis. The history indicated that symptoms of depression occurred around the 4th month of age, with slow and progressive emaciation. The ground where the pigeons were raised was heavily infested with pillbugs (*Armadillidium* spp.) and sowbugs (*Porcellio* spp.). No acute deaths were reported by the owner. On necropsy the prominent lesion consisted of an enlargement of the proventriculus, which was approximately 50 mm in length and 40 mm at its widest point (figure 1).

Dark red and bluish round areas 4 to 5 mm in diameter were also visible through the serosal surface of the proventriculus. With the exception of a mild degree of general atrophy of the liver, the other organs appeared normal. Macroscopically the proventricular wall was quite thickened, and the mucosal

Fig. 1. A. americana infection in domestic pigeon. Note the enlarged proventriculus and the dark areas each representing one female parasite X2 (Photo by L. H. Hardaker).

surface was covered with fibrin which nearly obliterated the lumen (figure 2). All birds presented 15 to 30 dark red bodies embedded in the proventricular wall and their presence was indicated by a red pointed appendage which protruded from the affected glands into the lumen of the organ. By dissection,

it was possible to liberate an organism approximately 3 mm in diameter, globular in shape, blood red in color, with four longitudinal furrows which cut deeply into the body of the parasite (figure 3). On histopathological examination, the

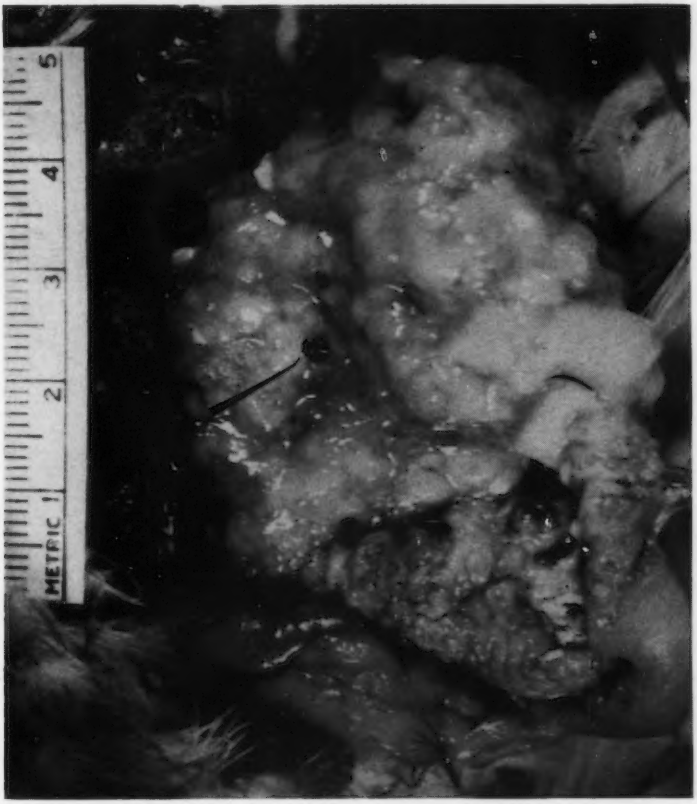

Fig. 2. T. americana infection in domestic pigeon. The enlarged proventriculus was opened to expose the mucosa. The arrow points to a female parasite dissected out of a gland X1.7 (Photo by L. H. Hardaker).

parasites were found at the bottom of the proventricular glands (figure 4), causing pressure atrophy of the acini without evidence of necrosis in the adjacent tissues. Sections of

the parasite and embryonated ova were also observed in the lobules of the deep glands (figures 5 and 6). The stroma of the proventriculus was infiltrated with leukocytes and a con-

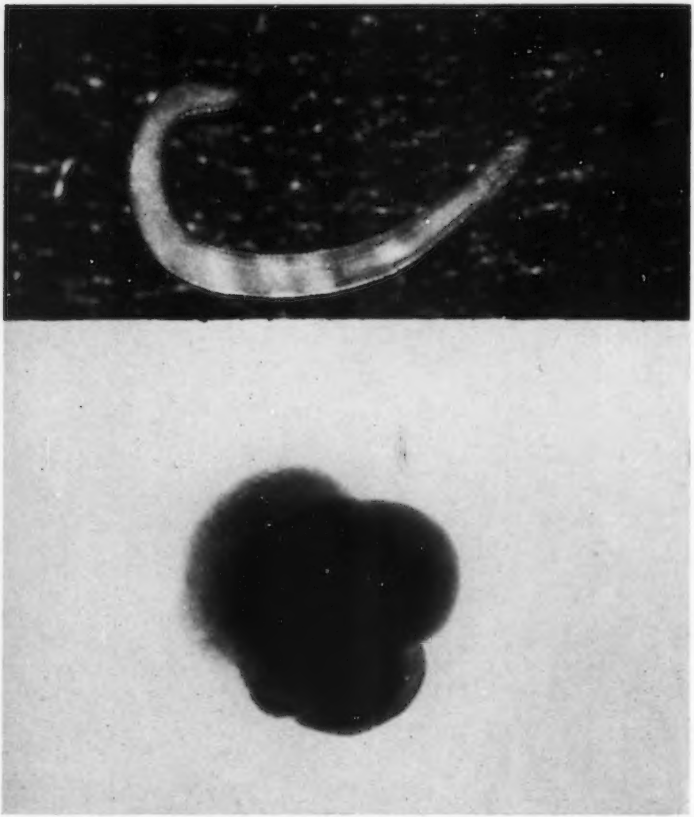

Fig. 3. D. nasuta (above) and *T. americana* (below) from the same pigeon. The latter worm is blood-red in color and the darker areas represent the intestine. X10. (Photo by L. H. Hardaker).

siderable amount of mucus was observed in the lumen of the glands. The serosal surface, the muscularis mucosae and the muscular tunic of the proventriculus appeared normal. Typical

appearance of the parasite and location of the lesion suggested a presumptive diagnosis of *T. americana* infection.

Buried in the glands of the same organ and visible because of their protruding tails, were filiform parasites approximately 10 mm in length and less than 0.5 mm in diameter.

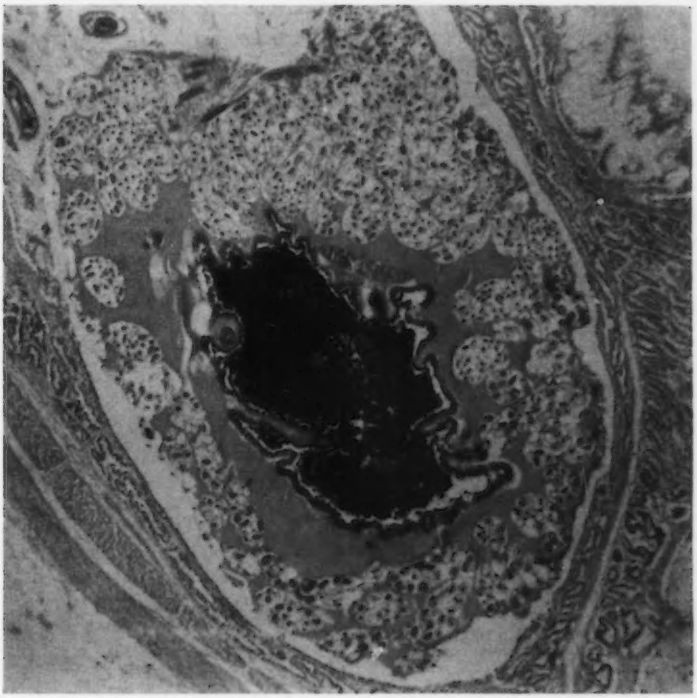

Fig. 4. Section of *T. americana* at the bottom of a proventricular gland. The dark center is the intestine surounded by the uterus containing ova. X44 (Photo by L. H. Hardaker).

These were tentatively classified as *Dispharynx nasuta*. Specimens of the two parasites were sent to the Animal Disease and Parasite Research Branch of the United States Department of Agriculture in Beltsville, Maryland, and were identified as *T. americana* and *D. nasuta*.*

T. americana and *D. nasuta* are both nematodes belong-

* The cooperation of Dr. E. E. Wehr in confirming the identity of the two parasites is kindly acknowledged.

ing to the superfamily Spiruroidea. This superfamily is characterized by an esophagus consisting of a muscular anterior portion and a longer glandular posterior portion. The life cycle as a rule requires arthropods as intermediate hosts.

T. americana shows marked sexual dimorphism. The males measure 5 to 6 mm in length and are very slender. The

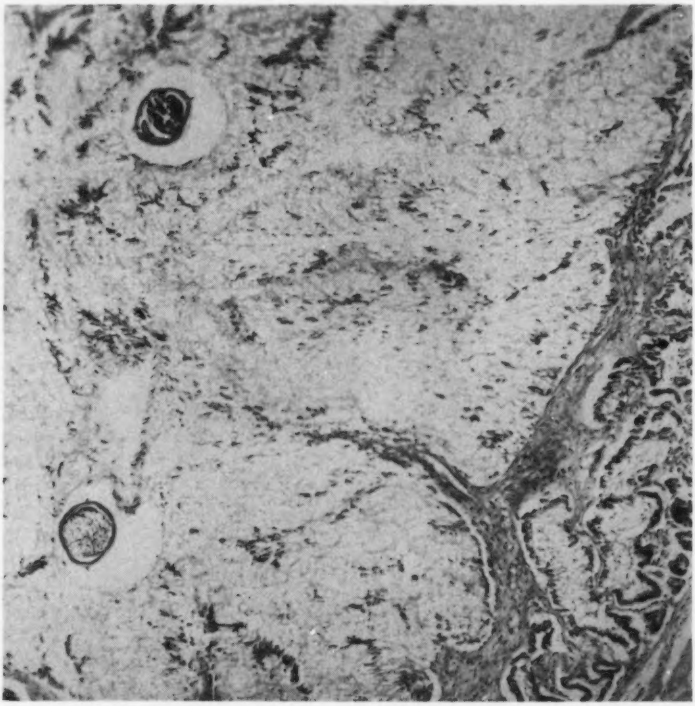

Fig. 5. Sections of *Tetrameres* present in the glands of the proventriculus of a domestic pigeon. X92 (Photo by L. H. Hardaker).

spicules are unequal in length with the longer measuring 300μ and the shorter 100μ. The females are approximately 4.0 mm in length and 3.0 mm in width. The body is blood red in color with four deep longitudinal furrows. Certain species of grasshoppers and a cockroach have been shown by Cram[3] to act as intermediate hosts for *T. americana* in the United States.

D. nasuta shows little sexual dimorphism. Both sexes are slender and have four wavy, cuticular cordons originating at the anterior end and passing backward. The cordons are recurrent but do not anastomose. The males measure from 5 to 8 mm in length while the females measure from 5.5 to 10 mm. The spicules are unequal in length and different in shape:

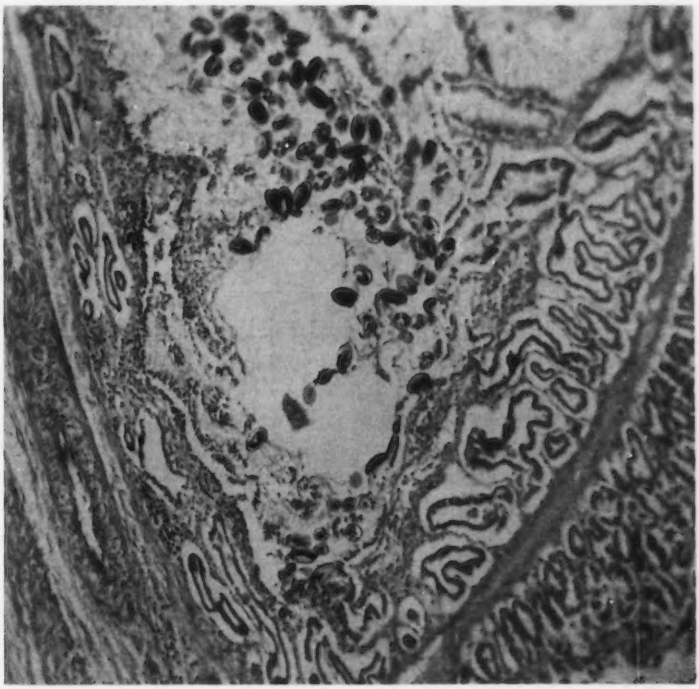

Fig. 6. Ova of *Tetrameres* in the glands of the proventriculus of a domestic pigeon. X89 (Photo by L. H. Hardaker).

the left is slender and 400 to 500 μ long, the right is boat-shaped and 150 to 200 μ in length.

The pillbug (*Armadillidium* spp.) and sowbug (*Porcellio* spp.) are capable of serving as intermediate hosts to these parasites. Since no specific treatment is available for either of the two parasites, it was decided to attempt control by

elimination of the intermediate hosts. This was accomplished by purchasing young chickens and allowing them to feed on the infested ground. This procedure apparently resulted in a sufficient reduction of the pillbugs and sowbugs to adequately control the spread of the parasites.

SUMMARY

This is the first of two natural outbreaks of *Tetrameres americana* and *Dispharynx nasuta* in domestic pigeons diagnosed in this laboratory. It indicates the possibility that the infection is more common in nature than realized and may be of practical significance to persons dealing with the production or diseases of pigeons.

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THE PATHOGENESIS OF THE ENCEPHALITIS IN TURKEY POULTS PRODUCED BY A NEUROTROPIC PLEUROPNEUMONIA-LIKE ORGANISM

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THE pleuropneumonia-like organisms (PPLO) are capable of producing a variety of pathological processes in chickens and turkeys. In addition to aerocystitis, tracheitis and sinusitis[10], inflammatory changes due to these organisms have been observed in the joints[11] and brain.[6] Encephalitis was described by Minard and Jungherr[6] and by Groupe, Winn and Jungherr.[4] They succeeded in producing the disease in chicks and poults that had been inoculated with the then poorly characterized infectious sinusitis agent. Delaplane[2] found that the sinusitis agent produced nervous symptoms in some poults as a result of infection in the infraorbital sinus.

Three years ago a PPLO was isolated here at the School of Veterinary Medicine by Zander from the brain of a turkey exhibiting encephalitis symptoms. Subsequent experiments indicated that this strain was consistently neurotropic when inoculated intravenously in turkeys. In view of these observations the following experiment was undertaken to study the pathogenesis of PPLO-induced encephalitis of turkeys.

EXPERIMENTAL PROCEDURE

Thirty-four Broad Breasted Bronze poults aged seven weeks were used in this experiment. Sera from these birds did

not have agglutinins for a standard PPLO antigen. The poults were inoculated intravenously with 1.0 ml of a fifth-generation broth culture passage of the neurotropic strain of PPLO. The inoculum was prepared from a 24-hour culture grown in PPLO broth (Difco) supplemented with ten percent horse serum and one percent yeast hydrolysate.

On each of the seven days following inoculation, from two to five birds were destroyed and necropsied. On the 12th and 15th day one and four birds were necropsied respectively (table I.) The brains were fixed by immersion in ten percent neutral formalin. Entire transverse sections were prepared from four levels: middle of forebrain, thalamus with optic tracts, midbrain through the middle of the optic tecta, and through the cerebellar and pontile area. Blocks were embedded in paraffin and routinely stained with hematoxylin and eosin. Cresyl violet, Holzer, Gomori modification of Bielschowsky reticulin, Giemsa and Oil Red O methods were also employed on selected tissues.

RESULTS

Symptoms. The first nervous symptoms as a result of intravenous inoculation of PPLO were observed on the third day. Five birds in the group had symptoms of torticollis and showed a marked state of excitability. The remaining poults showed only hyperesthesia. From the third day onward the symptoms generally progressed from hyperactivity to torticollis and finally somnolence. In the last stage the poults exhibited marked leg weakness and tended to rest on their hocks. The survivors were rough-feathered but did not have nervous signs.

No gross lesions were observed in any of the poults through the first 12 days. In the last group destroyed at 15 days post-inoculation, the birds had diffuse aerocystitis, one bird had obvious brain lesions. Table I shows the sites, time of appearance and frequency of microscopic focal necrosis. No necrotic foci were found on the 1st day. On the 2nd and 3rd days 40 to 50 percent of the birds had one or two foci each, all less than 0.5 mm in diameter. On the 4th and 5th days

60 to 80 percent averaged 2.4 foci each. From the sixth day on, all birds averaged seven necrotic foci each. May of these foci were 1 mm in diameter and one reached 2 by 4 mm. There was no correlation between type and degree of nervous symptoms and the site, size or severity of vascular and necrotic lesions. Several of the birds with minimal symptoms had the most severe damage.

Cultural results. PPLO were recovered in small numbers from the brains of the inoculated poults at the 24 and 48-hour periods. Large numbers of PPLO were present in the brains from the 3rd to the 7th day. The organism was not recovered from the poults destroyed on the 12th day and from only one of the five poults cultured on the 15th day. An interesting observation concerning this organism was that the brain tissue did not inhibit growth of the organism as is frequently the case with many bacteria. PPLO colonies on agar grew profusely beneath the smeared brain tissue.

Pathogenesis. The five birds killed one day after inoculation showed meager lesions, chiefly consisting of small microglial foci or nodules with a few free mesodermal cells (fig. 1). These foci were not associated with larger vessels. Endothelial swelling and some proliferation were apparent. A moderate degree of hyperemia was present. No necrotic foci were found. Neurons showed only a little vacuolation and chromatolysis. Except for some hyperemia the leptomeninges showed no abnormality.

Glia nodules were larger, but no more numerous on the 2nd and 3rd days. Striking at this stage were the vasoproliferative changes. Vessels of all sizes were affected erratically, but often severely. Endothelial hyperplasia was frequent, leading to some reduction in caliber of lumen. Even more marked was the increase in adventitial cells. These were not seen as typical perivascular infiltrations, but as rather broad adventitial zones of polygonal or stellate cells with relatively abundant, pink cytoplasm and large, pale, oval nuclei. The cells resembled macrophages and occasionally showed mitotic figures. Rarely a lymphocyte or granulocyte was observed in the vessel wall. Thrombosis was not encountered.

Four of these nine birds showed occasional tiny (less than 0.5 mm) foci of necrosis without cellular reaction in forebrain or midbrain (table I). These were presumed to be due to occlusive vascular lesions.

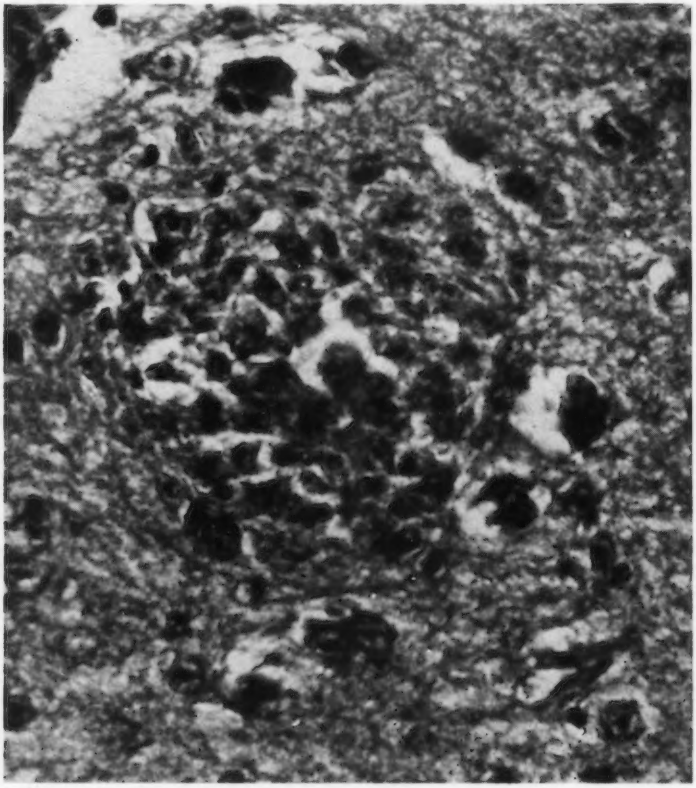

Fig. 1. Small parenchymal nodule in brain of turkey killed on the 2nd day of infection. A few free mesodermal cells are present.

At this stage meningitis was first distinctly evident. There was a diffuse accumulation of macrophages with some lymphocytes and granulocytes. Hyperemia and edema were present but not marked. Infrequently a leptomeningeal vessel showed proliferative and infiltrative changes and adventitial deposits of fibrinoid material.

TABLE I

The sites, time of appearance and frequency of microscopic focal necrosis in the brains of turkeys inoculated intraveneously with a neurotropic PPLO.

Day post-inoc.	1	2	3	4	5	6	7	12	15	Total
No. of birds	5	5	4	5	5	3	2	1	4	34
Birds with one or more foci	0	2	2	3	4	3	2	1	4	21
Forebrain	0	0	1	3	9	7	8	2	21	51
Thalamus	0	0	0	1	2	4	3	2	6	18
Midbrain	0	3	1	2	0	5	4	1	5	21
Cerebellum	0	0	0	0	0	2	0	0	0	2
Total	0	3	2	6	11	18	15	5	32	92
Av. foci/bird*	0	1.5	1	2	2.7	6	7.5	5	8	4.4

*Average number of foci in 4 sections per affected bird.

On the 4th and 5th days both the vasculitis and meningitis became progressively more severe. Parenchymal vessels showed more and larger lesions, with larger numbers of lymphocytes and frequent plasma cells (fig. 2). Leucocytes could be seen throughout the thickness of the vessel walls with accompanying degenerative changes. Reticulin fibers were forced apart and showed some fragmentation and irregular thickening. Many fine droplets which stained a brilliant red with Oil Red O were seen in the cytoplasm of macrophages and occasionally in endothelium.

Necrotic foci, some up to 1 mm in diameter, were more frequent but still showed no cellular reaction. Forebrain foci were more numerous than those at thalamic or midbrain levels.

The meningeal reaction was much more severe than on the second or third day. The vessels showed striking involvement and fibrinoid material was common.

Cases from the 6th and 7th days showed parenchymal vasculitis of a type and severity comparable to that of the two previous days. There did appear to be more degenerative change in the vessel walls, and a few thrombi were found. Plasma cells about the vessels seemed to be somewhat more numerous.

All of these birds showed necrotic foci, and these were

larger and more numerous (fig. 3). On the 7th day, a few foci showed microglial activity with phagocytosis of peripheral necrotic material. No cellular reaction was present about most of the foci, however.

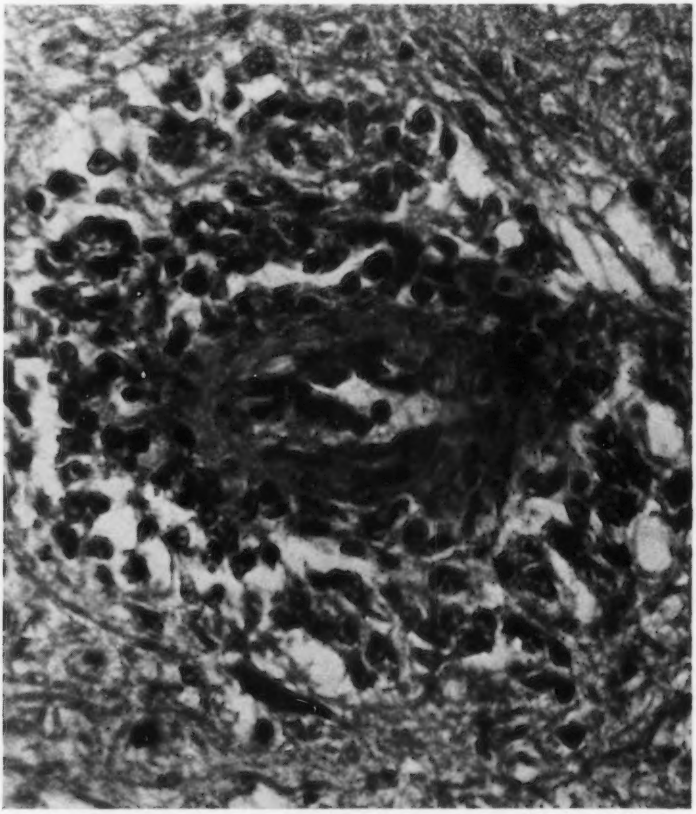

Fig. 2. Parenchymal vessel showing endothelial proliferation and cells in adventitia on the fifth day of infection.

The meningeal reaction, especially the vascular damage was at its height. Degenerative and infiltrative changes in the vessel wall was maximal. Thrombosis was occasionally observed.

The birds killed on the 12th and 15th days showed a distinct diminution of the parenchymal vascular reaction. While a few vessels still were severely affected, the general picture was one of reduced numbers of mobile cells and of mural repair.

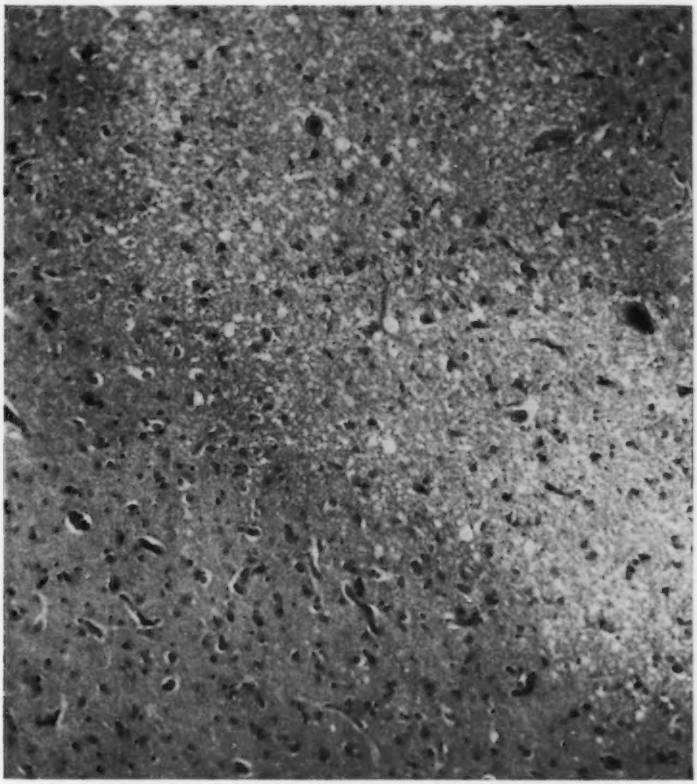

Fig. 3. Focal necrosis on the sixth day of infection.

Necrotic foci were somewhat more frequent than earlier, but nearly all foci showed reparative changes (fig. 4). Microglial phagocytes entirely filled the smaller foci and formed broad peripheral zones in the larger ones. Proliferation of

astrocytes was evident about the foci. Many foci had collapsed to some degree as necrotic tissue had been broken down.

The diffuse meningeal cellularity had largely disappeared, and excessive numbers of cells were apparent only in the ad-

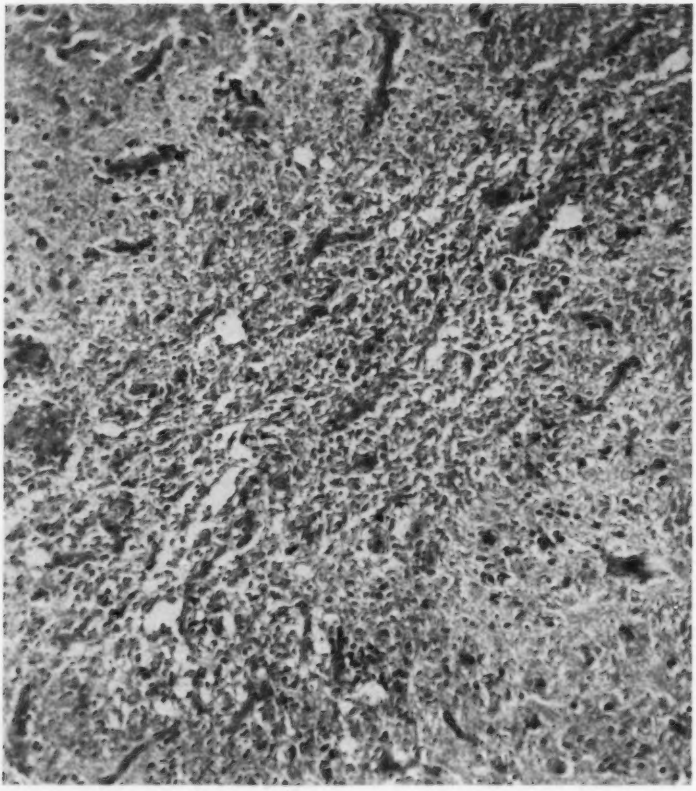

Fig. 4. Focal necrosis with beginning repair on the fifteenth day.

ventitia of vessels. This meningeal vasculitis was of extensive distribution but of decreasing severity. Fibrinoid material was rarely found. Adventitial and endothelial proliferation was still evident, and leucocytic infiltration was commonly seen in only a segment of the circumference of vessels.

Pattern of lesions. The glia nodules and parenchymal vasculitis, as well as the necrotic foci, were typically and consistently severe in some topographic areas and sparse in others. In the forebrain, the neostriatum and mesostriatum suffered greatest damage with relatively much less involvement of the paleostriatum, hippocampal formation and septum.

The thalamus was the seat of widespread lesions, but the large striofugal and thalamofrontal tracts and the most dorsal areas were distinctly least affected. The optic tracts showed necrosis in but one case, although vasculitis was frequent.

In the midbrain, the optic tectum showed very few lesions, most of the damage being found medial to the tectal ventricular cavities.

Cerebellar and pontile lesions were much less frequent and severe than at any of the higher levels. Vasculitis was of lesser degree and confined to the region of the pontile nuclei, cerebellar peduncles and cerebellar white substance. A single bird showed necrosis at this level, bilaterally symetrical foci being found in the cerebellar peduncles.

A pallor and loosening of the neuropil, without actual necrosis, was frequently observed immediately about the third ventricle and acqueduct, and in the cerebellar white substance.

The meningeal reaction was widespread, but most severe basally and over the thalamus and midbrain.

DISCUSSION

Spread of the infection to the brain was undoubtedly hematogenous, as indicated by the marked vasculitis and the absence of extension from meninges, ventricle system or nerve roots. The meningitis appeared and reached its peak later than the parenchymal process. The reaction appeared to affect capillaries first, but the marked lesions of small arteries and even veins soon came to dominate the picture.

The vasculitis, both parenchymal and meningeal, was the characteristic feature of the disease. The extent of endothelial and adventitial proliferation was a striking phenomenon. Leucocytic infiltration was widespread in later stages, but

discrete accumulations in the perivascular spaces were not typical. Leucocytes, chiefly lymphocytes and plasma cells, were found throughout the wall and periadventitially. Fibrinoid material was abundantly present in the outer layers at 4 to 7 days in meningeal vessels, but was never very abundant in parenchymal vessels. Necrosis and thrombosis were not frequent in either site. Endothelial swelling and proliferation at times nearly or entirely obliterated the lumen, especially in vessels of the parenchyma.

The focal necrosis was presumed to be due to vascular occlusion rather than to action of toxins directly. Like the vascular damage, it was erratically distributed but widespread and in the same areas. Until the 7th day, the necrotic tissue showed no cellular reaction. Beginning at this time a progressive reparative process appeared.

There is very little in the literature on PPLO lesions in the nervous system of any host species. Rolling disease in mice has been described by Findlay and co-workers[3] and by Sabin.[8,9] The former investigators reported the presence of large numbers of polymorphonuclear leucocytes in the parenchyma and equal numbers of these and mononuclear leucocytes in the meninges. Sabin mentions acute cerebellar necrosis and disintegration of structures about the lateral ventricles with intracerebral inoculation and widespread neuronal degeneration after intraperitoneal or intrathoracic inoculation. Mooser[7] produced a similar entity in mice with his PPLO strain but regarded the neuronal necrosis as more probably due to vasospasm than to a neurolytic toxin.

The lesions reported here in poults are very similar to those seen by Jungherr[5] working with two strains of a nonfiltrable agent isolated from cases of sinusitis and of aerocystitis in turkeys. His strains, now presumed to be PPLO, produced nervous symptoms in half of the poults and chicks inoculated by intracerebral or other parenteral routes, except intraocular. He reported an endarteritis of arterioles and medium-sized muscular arteries with hypertrophy and hyperplasia of endothelium, necrosis and hyaline degeneration of the media and a few vascular and perivascular infiltrating

cells. The accompanying foci of rarefaction or frank encephalomalacia were thought to have a basis in vascular occlusion. Leptomeningeal vessels showed a similar change with occasional thrombosis and some diffuse meningeal infiltration. Lesions were of variable intensity and distribution, but periventricular areas seemed to be most frequently involved.

SUMMARY

The development of an encephalitis in turkeys produced by a highly neurotropic PPLO is described. The disease is characterized by vasculitis and focal necrosis.

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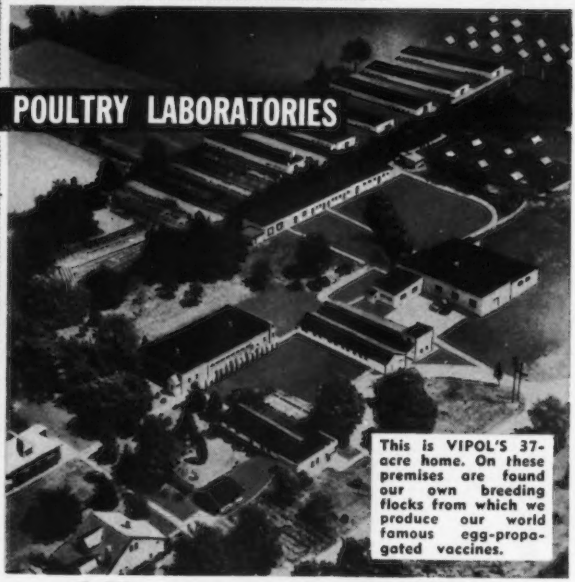

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